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Sludge reduction using the oxic-settling-anoxic (OSA) process: underlying mechanisms, microbial community, and fate of trace organic contaminants

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CERTIFICATION

I, Galilee Uy Semblante, hereby declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy to the School of Civil, Mining and Environmental Engineering, Faculty of Engineering and Information Sciences, University of Wollongong, is wholly my own work unless otherwise acknowledged. The document has not been submitted for qualification at any other academic institution.

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THESIS-RELATED PUBLICATIONS

1. **Semblante, G.U.**, Hai, F.I., Ngo, H.H., Guo, W., You, S.-J., Price, W.E., Nghiem, L.D. 2014. Sludge cycling between aerobic, anoxic and anaerobic regimes to reduce sludge production during wastewater treatment: Performance, mechanisms, and implications. *Bioresource Technology*, 155, 395-409.
2. **Semblante, G.U.**, Hai, F.I., Bustamante, H., Guevara, N., Price, W.E., Nghiem, L.D. 2015. Effects of iron salt addition on biosolids reduction by oxic-settling-anoxic (OSA) process. *International Biodeterioration & Biodegradation*, 104, 391-400.
3. **Semblante, G.U.**, Hai, F.I., Huang, X., Ball, A.S., Price, W.E., Nghiem, L.D. 2015. Trace organic contaminants in biosolids: Impact of conventional wastewater and sludge processing technologies and emerging alternatives. *Journal of Hazardous Materials*, 300, 1-17.
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8. **Semblante, G.U.**, Hai, F.I., McDonald, J., Khan S.J., Nelson, M., Lee, D.J., Price, W.E., Nghiem, L.D. 2017. Fate of trace organic contaminants during sequencing batch reactor treatment coupled with oxic-settling-anoxic (OSA) for biosolids reduction. *Bioresource Technology* (In press).

ABSTRACT

The conventional activated sludge (CAS) process is widely used for treating domestic and industrial wastewaters. However, it produces large amounts of excess sludge that requires treatment, handling, and disposal. These procedures represent a major fraction of the total operating cost of wastewater treatment plants (WWTPs). Furthermore, processed sludge called "biosolids" that are applied in agriculture may contain trace organic contaminants (TrOCs) that adversely affect the environment and human health. To reduce the cost of sludge management and the environmental risks associated with residual sludge, a strategy that will minimise sludge production must be formulated. The oxic-settling-anoxic (OSA) process is a promising approach to minimise sludge production. OSA modifies CAS by placing external anoxic reactor/s in the return activated sludge (RAS) loop. The interchange of sludge between conditions that are rich (the aeration tank) and deficient (the external anoxic reactor/s) in oxygen and substrate has been found to result in net excess sludge reduction. Due to its simple design, OSA has relatively low capital and maintenance cost. However, the promising sludge reduction rates observed in laboratory-scale OSA operated with synthetic wastewater have not been realised in pilot- or full-scale implementations. This is due to knowledge gaps in the fundamental operation of OSA. The mechanistic process of OSA, especially the factors affecting OSA performance and the role of microbial community structure on sludge reduction, are poorly understood. Moreover, the occurrence of TrOCs in residual sludge produced by OSA has not been evaluated.

The overreaching goal of this study is to obtain a comprehensive understanding of the OSA process. This study aims to determine the effect of three factors – iron salt dosage, sludge interchange rate (SIR), and sludge retention time (SRT) – on sludge reduction. Applying an integrative approach that focuses on these factors will help elucidate the underlying mechanisms governing sludge reduction. Additionally, this study aims to investigate the fate of TrOCs (*i.e.*, occurrence, sorption, and biodegradation) in OSA. In this study, a laboratory-scale OSA system consisting of a sequencing batch reactor (SBR) attached to external intermittently aerated (*i.e.*, aerobic/anoxic) and anoxic reactors was operated. The extent of sludge reduction was assessed by comparing OSA with a control system consisting of an SBR attached to a single-pass aerobic digester. The two systems were operated in parallel using real wastewater. Using real wastewater

is crucial to this study because it helped cultivate biomass with realistic growth rates and properties.

Results showed that the OSA and control systems produced effluent with similar effluent quality in terms of chemical oxygen demand (COD), ammonia, and orthophosphate concentrations. However, OSA achieved more than 35% sludge yield reduction depending on operation conditions (iron salt dosage, SIR, and SRT).

Iron salts are commonly added to the influent for phosphorous removal. However, this study showed that iron salt ($FeCl_2$) was counterproductive to sludge reduction in the external aerobic/anoxic reactor of OSA. The concentration of extracellular polymeric substances (EPS) in the aforementioned reactor increased when $FeCl_2$ was dosed to the influent. $FeCl_2$ promoted sludge flocculation and consequently prevented the disintegration of EPS under aerobic/anoxic conditions. This study further demonstrated that the destruction of sludge flocs through EPS disintegration is a key mechanism for volatile solids reduction in the external reactors of OSA.

SIR is the percentage by volume of sludge interchanged between the main aeration tank (SBR) and external reactors of OSA. This study showed that an intermediate SIR (11%) resulted in optimum OSA performance through two mechanisms: (a) providing an environment that was conducive for volatile solids destruction as evidenced by the increase in orthophosphate under anoxic conditions; (b) facilitating the conversion of lysed materials into inert forms as evidenced by the decrease in ammonia and nitrate under aerobic/anoxic conditions. SIR of more than 11% resulted in lower sludge reduction, whereas without SIR sludge reduction in the main bioreactor cannot take place.

SRT_{ext}, *i.e.*, the SRT of the external reactors, have significant impact on OSA performance. This study showed that under optimum SRT_{ext} (20 d), OSA reduced sludge by facilitating volatile solids destruction in the external anoxic reactor and nitrification/denitrification in the external aerobic/anoxic reactor. Increasing SRT_{ext} facilitated the autolysis of sludge under oxygen- and substrate-deficient conditions. However, beyond the optimum SRT_{ext}, further biodegradation of sludge did not occur. Instead, nitrification/denitrification efficiency in the external aerobic/anoxic reactor decreased, and this consequently deteriorated OSA performance.

This study elucidated the mechanism of sludge reduction from a microbiological perspective. Specific bacteria such as *β*- and *γ-Proteobacteria* decayed due to lack of oxygen and substrate in the external reactors of OSA. Nonetheless, other microorganisms such as hydrolysing (*e.g.*, phyla *Bacteroidetes* and *Chloroflexi)*, fermentative (*e.g.*, orders OP8, *Firmicutes*, WS3, and *Spirochaetae)*, denitrifying (*e.g.*, *Xanthomondales*) and predatory (*e.g.*, orders *Myxobacteriales* and *Bdellovibrio)* bacteria proliferated in the external reactors of OSA. The increase in the abundance of predatory and denitrifying bacteria in the external reactors of OSA coincided with high sludge reduction under an optimum SRT_{ext} of 20 d. Predators probably facilitated sludge autolysis, while denitrifiers probably played a key role in converting destroyed volatile solids into inert forms.

This study determined the sorption and biodegradation of a total of 52 TrOCs in OSA. The wide range of TrOCs included pharmaceuticals, pesticides, and industrial chemicals that occurred in real wastewater. Results show that OSA did not negatively affect the effluent TrOC concentration of the SBR. In fact, a few TrOCs that were recalcitrant in the SBR (*e.g.*, benzotriazole) were highly biodegraded in the external aerobic/anoxic reactor. The OSA configuration used in this study discharges sludge from an aerobic/anoxic reactor rather than an anoxic reactor, which is commonly found in literature. Results show that the aerobic/anoxic treatment resulted in greater TrOC biodegradation than the anoxic treatment. Moreover, the destruction of volatile solids in the anoxic reactor caused desorption of some TrOCs (*e.g.*, paracetamol, sucralose, and bisphenol A) from the solid phase of sludge and consequently increased TrOC concentration in the aqueous phase. These findings suggest that the current OSA configuration has potential to have lower TrOC emission than others involving a single external anoxic reactor. Furthermore, the concentration of highly hydrophobic TrOCs (*e.g.*, triclosan and triclocarban) of the final sludge residue of the OSA system (sludge discharged from the aerobic/anoxic reactor) was lower than that of the control system (sludge discharged from the aerobic digester), suggesting that OSA can help reduce TrOCs in biosolids.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AOO	Ammonia-oxidizing organisms
ASSR	Anaerobic side-stream reactor
ATP	Adenosine triphosphate
CST	Capillary suction time
CAP	Constrained analysis of principal coordinates
CAS	Conventional activated sludge process
DO	Dissolved oxygen
DS	Dry solids
EPS	Extracellular polymeric products
FIA	Flow injection analysis
F/M	Food-to-microorganism ratio
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
ORP	Oxidation-reduction potential
OSA	Oxic-settling-anoxic process
OTU	Operational taxonomic unit
PAO	Polyphosphate-accumulating organisms

PCoA	Principal coordinate analysis
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
PD	Phylogenetic diversity
RAS	Return activated sludge
rRNA	Ribosomal ribonucleic acid
SBR	Sequencing batch reactor
sCOD	Soluble chemical oxygen demand
SIR	Sludge interchange rate
SMP	Soluble microbial products
SPE	Solid phase extraction
SPR	Sludge process reduction
SRT	Sludge retention time
SS	Suspended solids
SVI	Sludge volume index
tCOD	Total chemical oxygen demand
TQMS	Triple quadrupole mass spectrometry
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorous
TrOC	Trace organic contaminants
TSS	Total suspended solids

VSS	Volatile suspended solids
WAS	Waste activated sludge
WWTP	Wastewater treatment plant

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND OF THE STUDY

1.1.1 Overview of the activated sludge process

Activated sludge is the most widely-used process for treating domestic and industrial wastewaters. It involves a consortium of microorganisms that consume organic matter and nutrients in the influent for cell maintenance and propagation. After treatment, the biomass or "sludge" is allowed to settle to separate treated water (Wei *et al.*, 2003; Guo *et al.*, 2013). Wastewater treatment efficiency is significantly affected by the ratio of food and microorganisms (F/M), which is maintained by wasting excess sludge that accumulated in the bioreactor (Abbassi *et al.*, 2000; Wei *et al.*, 2003). Because sludge contains active (live) and inactive (dead) microorganisms, it must be adequately treated prior to disposal to prevent impact on the environment and public health. Sludge treatment involves several unit processes that reduce the mass, moisture, and pathogen content of sludge to ensure that the final residue can be transported and disposed with minimal cost and risk (Low and Chase, 1999; Wei *et al.*, 2003).

1.1.2 Excess sludge production in biological treatment

Although the activated sludge process can achieve high wastewater treatment efficiency, it produces large amounts of excess sludge that requires management and disposal (Wei *et al.*, 2003; Foladori *et al.*, 2010). Sludge production is continuously increasing worldwide due to population growth, industrialization, and enhancement of wastewater treatment coverage in response to public sanitary requirements (Wei *et al.*, 2003; Foladori *et al.*, 2010). The production of sludge in the European Union increased from 6 to 9 million tonnes dry solids (DS) within 1992-2005 (Neyens and Baeyens, 2003). The United States generated 6.9-7.6 million tonnes DS per year in the period of 2005-2010 (Foladori *et al.*, 2010). The highest production is observed in China, which generated 11.2 million tonnes DS in 2010 (Neyens and Baeyens, 2003). In Australia, sludge production increased by about 3% each year from 0.30 million tonnes DS in 2010 to 0.33 million tonnes in 2013 (Foladori *et al.*, 2010).

The cost of sludge treatment and disposal represent a major fraction (up to 60%) of the total operating cost of wastewater treatment plants (WWTPs) (Zhang *et al.*, 2007; Foladori *et al.*, 2010). Treatment procedures can be technically challenging due to the inherent properties of the biomass (Mahmood and Elliott, 2006). For instance, "dewatering" or the process of decreasing

the water content of sludge to 60% is encumbered by the fact that biopolymers (*i.e.*, proteins and polysaccharides) have high affinity towards water molecules (Chu *et al.*, 2009). Furthermore, there are limited options for sludge disposal. Traditional means such as ocean-dumping and land-filling were banned to protect marine life and restricted due to high cost of maintenance, respectively (Saby *et al.*, 2002; Foladori *et al.*, 2010). Other practices, namely sludge incineration and re-use, have disadvantages as well. Incineration decreases sludge volume by 95%, but it consumes large amounts of energy (Clarke and Smith, 2011). The beneficial re-use of high-quality treated sludge called "biosolids" is recently gaining emphasis because of its potential to add economic value to residual sludge. Biosolids is rich in carbon (55%), nitrogen (15%), and phosphorous (3%), and can be utilised as soil conditioner or fertiliser (Ghyoot and Verstraete, 2000). However, converting sludge into a form that meets the strict standards for land application (*e.g.*, very low levels of disease-causing vector attraction and heavy metal concentration) can be an arduous and expensive exercise (Wei *et al.*, 2003). Additionally, not all farmlands can receive biosolids because several factors such as soil chemistry, proximity to residential areas, and accessibility must be considered (Chen *et al.*, 2003; Goel and Noguera, 2006). Further concern emerges as biosolids may accumulate trace organic contaminants (TrOCs), which are pharmaceuticals, personal care products, hormones, pesticides, and industrial chemicals that are recalcitrant to biological wastewater and sludge treatment. Certain TrOCs disrupt the endocrine system and may cause physical abnormalities and reproductive problems in animals and humans (Camacho *et al.*, 2005; Foladori *et al.*, 2010; Fukahori and Fujiwara, 2015).

The abovementioned issues underscore the fact that excess sludge production is one of the most vexing problems in WWTPs. It is imperative to formulate strategies in the area of sludge management that will promote cleaner production, *i.e.*, the decrease of sludge production as well as the energy and costs associated with sludge disposal. The most efficient way to achieve this is to minimise the amount of sludge that is wasted during wastewater treatment. As a general rule, preventing the creation of waste products is desired to decrease the efforts exerted towards waste handling and disposal. In other words, reducing sludge production is more economical than dealing with sludge that has already been produced (Canales *et al.*, 1994; Neyens and Baeyens, 2003).

1.1.3 Various approaches to reduce sludge production

Sludge production can be minimised through several approaches, namely, control of operating parameters, destruction of sludge by physical, thermal, or advanced oxidation processes (Rocher *et al.*, 2001; Liu, 2003; Neyens and Baeyens, 2003; Chu *et al.*, 2009), addition of chemicals that disrupt biomass growth (Zhang *et al.*, 2007; Vaxelaire *et al.*, 2008), and alternating redox conditions (aerobic, anoxic, and anaerobic sludge cycling regimes) (Ahn *et al.*, 2002). Controlling parameters such as increasing sludge retention time (SRT) and dissolved oxygen (DO) concentration can only result in marginal improvement but may increase plant operation costs (Yasui *et al.*, 1996). Sludge destruction greatly reduces sludge production, but this approach requires high capital investment and on-going maintenance (Kamiya and Hirotsuji, 1998; Chen *et al.*, 2001; Neyens and Baeyens, 2003). Adding chemicals or using advanced oxidation processes can introduce potential contaminants to the sludge and effluent streams (Mahmood and Elliott, 2006). Cycling between different redox conditions is arguably the most benign and cost-effective approach to minimise sludge production. This technique was pioneered by Westgarth et al. (2010), who inserted an anaerobic tank in the return sludge line of a conventional activated sludge (CAS) system and observed 50% decrease in sludge production. Contemporary researchers adapted the design and coined the term "oxic-settling-anoxic" or OSA process (Wei *et al.*, 2003; Foladori *et al.*, 2010).

1.1.4 Sludge reduction using OSA

OSA modifies CAS (Figure 1.1a) by placing external anoxic reactor/s in the return activated sludge (RAS) loop (Figure 1.1b). Sludge is partially biodegraded in the external reactor/s, which is low in DO (*i.e.*, under anoxic or anaerobic condition) and substrate concentration, before it is returned to the aeration tank. The interchange of sludge between conditions that are rich (the aeration tank) and deficient (the external anoxic reactor/s) in oxygen and substrate results in net excess sludge reduction (Semblante et al., 2014). Due to its simple design, it is feasible to set up OSA in existing or new WWTPs using readily available equipment (*e.g.*, tanks, tubings, and pumps). Furthermore, OSA requires minimal maintenance in comparison with other sludge

minimisation techniques that require complex machinery (*e.g.*, advanced oxidation processes) (Saby *et al.*, 2002; Mahmood and Elliott, 2006; Foladori *et al.*, 2010).

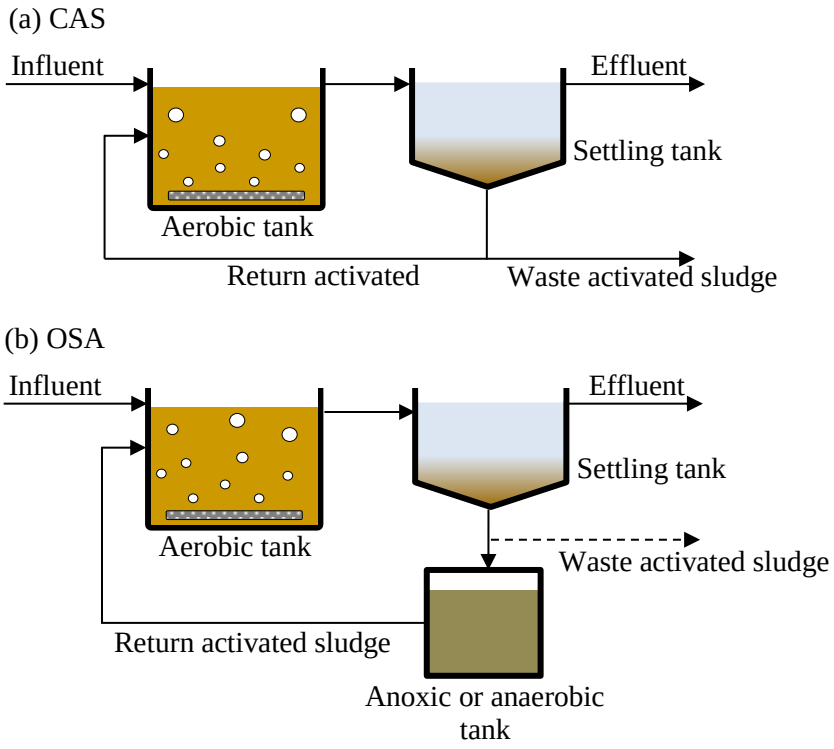

Figure 1.1. Schematic diagram of (a) CAS and (b) OSA processes

1.2 STATEMENT OF THE PROBLEM

OSA and other processes with similar configurations have been implemented in WWTPs in Australia and other countries (Saby *et al.*, 2002; Liu, 2003; Foladori *et al.*, 2010). However, the promising sludge reduction rates (*e.g.*, 50-80%) observed in laboratory-scale systems fed with synthetic wastewater (Low and Chase, 1998; Chen *et al.*, 2002; Yang *et al.*, 2003) have not been realised in pilot- or full-scale systems, which only achieve up to 20% sludge reduction (Clarke and Smith, 2011; USEPA, 2013). This discrepancy is brought about by significant gaps of knowledge in the fundamental operation of OSA.

First, the underlying mechanisms responsible for sludge reduction in OSA are unclear (Ghyoot and Verstraete, 2000). Several mechanisms have been hypothesized such as the enhancement of lysis-cryptic growth, degradation of extracellular polymeric substance (EPS), and selection of

slow-growing bacteria (Lee and Welander, 1996; Elissen *et al.*, 2006). Evidence supporting these mechanisms is mostly based on laboratory-scale studies that have strictly controlled and potentially unrealistic conditions (*e.g.*, consistent influent strength and composition due to use of synthetic wastewater). Indeed, bioreactors fed with synthetic wastewater have different sludge yield and wastewater treatment efficiency than those fed with real wastewater (Paul *et al.*, 2001; Rene *et al.*, 2008). Therefore, studies that exclusively used synthetic wastewater may have cause over-estimated OSA performance.

Second, there is insufficient knowledge regarding the operation parameters that potentially have critical impact on OSA performance: iron salt dosage, sludge interchange rate (SIR), and SRT.

(a) Iron salts are commonly added to wastewater in full-scale plants for phosphorous removal by chemical process (Tamis *et al.*, 2011). It was reported that high iron dosing enhances OSA performance (Ghyoot and Verstraete, 2000). However, iron is known to bind with EPS (Wei *et al.*, 2003) and prevents sludge floc degradation (Foladori *et al.*, 2010; Semblante *et al.*, 2014). Thus far, the effect of iron on sludge reduction in OSA remains unclear.

(b) SIR is the percentage by volume of sludge returned from the external reactor to the main aeration tank of OSA. In literature, OSA is usually operated at an SIR of 10% (Saby *et al.*, 2003; Sun *et al.*, 2010; Chon *et al.*, 2011b). Some reports suggest that holding sludge in anoxic condition promotes its biodegradation (Troiani *et al.*, 2011; Coma *et al.*, 2013), but there was a study suggesting that short but frequent exposure to anoxic condition enhanced OSA performance (Semblante *et al.*, 2014).

(c) SRT of the external reactors can critically impact sludge reduction. However, there is a wide spectrum of SRTs and conflicting results in literature. OSA was conventionally operated at long SRT (30-80 d) to encourage sludge biodegradation (Saby *et al.*, 2002; Foladori *et al.*, 2010),but recent studies showed that short SRT (<30 d) resulted in appreciable sludge reduction at potentially less aeration cost (Novak *et al.*, 2007; Coma *et al.*, 2013).

Clearly, the few studies that are available have contradicting findings regarding the effects of the aforementioned parameters on sludge reduction. Moreover, the studies have different reactor configuration, operation condition, and method of quantifying sludge reduction (Liu and Tay, 2001; Chon *et al.*, 2011a; Niu *et al.*, 2016). Therefore, it is difficult to compare results across

various studies and to develop effective control strategies for OSA operation using current literature.

Third, the microbial community structure of OSA is not fully understood. Microbial communities are a rarely-studied but important but aspect of biological systems because they are closely linked to metabolic reactions and microbial interactions (Liu and Tay, 2001; Saby *et al.*, 2003). Previous studies observed that OSA has higher microbial diversity than CAS and hypothesised that certain microbial groups possibly drive sludge reduction (Chudoba *et al.*, 1992; Novak *et al.*, 2007). Most of these findings were derived from low-throughput techniques, such as 16sRNA sequencing and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), that provide inadequate information on microbial diversity and taxonomic classifications. It is only recently that high-throughput techniques such as pyrosequencing and Illumina sequencing are applied in OSA to gain a more in-depth perspective of microbial communities (Wang *et al.*, 2008; Ye *et al.*, 2008). Nevertheless, further research is necessary to identify the bacterial groups that are relevant to OSA performance and to understand the changes in microbial diversity due to operation conditions. Addressing these knowledge gaps will be useful in the design and optimisation of the OSA process.

Fourth, the fate of TrOC in OSA has not been investigated. Significant research efforts have been devoted to track the sorption and biodegradation pathways of TrOCs in CAS and other wastewater treatment systems (Chen *et al.*, 2003; Saby *et al.*, 2003). The occurrence of TrOCs in either effluent or biosolids results in the emission of contaminants in receiving water bodies, agricultural land, or groundwater and poses considerable environmental and health risks (An and Chen, 2008). It is worthwhile to understand TrOC sorption and biodegradation under operation conditions unique to OSA (*e.g.*, interchange of sludge between reactors that are rich and deficient in oxygen and substrate) as it will contribute to the body of knowledge on the physico-chemical interactions of organic contaminants and metabolic reactions in sludge.

1.3 OBJECTIVES OF THE STUDY

The primary goal of this study is to gain a thorough understanding of the fundamental mechanisms and factors affecting the performance of OSA. The specific objectives are as follows:

(i) To elucidate the underlying mechanisms responsible for sludge reduction in a laboratory-scale OSA fed with domestic sewage. The use of domestic sewage (*i.e.*, real wastewater) is critical because it will cultivate biomass with realistic growth rates and properties. Thus, this approach will provide practical insights on OSA mechanisms.

(ii) To systematically determine the impact of three operation parameters (iron salt dosage, SIR, and SRT) on OSA performance and to explain their effect on sludge reduction mechanisms.

(iii) To characterise the microbial community structure of OSA using Illumina sequencing, a high-throughput analytical technique, and to determine the role of microbial communities in sludge reduction.

(iv) To determine the fate of TrOCs in OSA, focusing on the effect of alternating redox conditions on TrOC sorption and biodegradation.

1.4 THESIS OUTLINE

This thesis is divided into nine chapters (Figure 1.2). Chapter 1 introduces the background and objectives of this study. Chapter 2 provides a comprehensive literature review of the state-of-the-art of OSA, concentrating on various OSA configurations and performance, mechanisms of sludge reduction, microbial community structure, and fate of TrOC. Chapter 3 provides a detailed account of the methodologies utilised in this study, including reactor configuration and analytical techniques. The results of this research are presented in five chapters. Chapters 4, 5, and 6 discuss the effects of iron salt dosage, SIR, and SRT, respectively, on OSA performance and their ramifications on the underlying mechanisms of sludge reduction. Chapter 7 discusses the microbial community structure of OSA with focus on the interrelation of microbial diversity, specific bacterial groups, and sludge reduction. Chapter 8 discusses the fate of TrOC in OSA with focus on TrOC sorption and biodegradation at different redox conditions. Finally, chapter 9 summarises the key outcomes of this research, the relevance and contribution of this research to the wastewater treatment industry, and recommendations for future study.

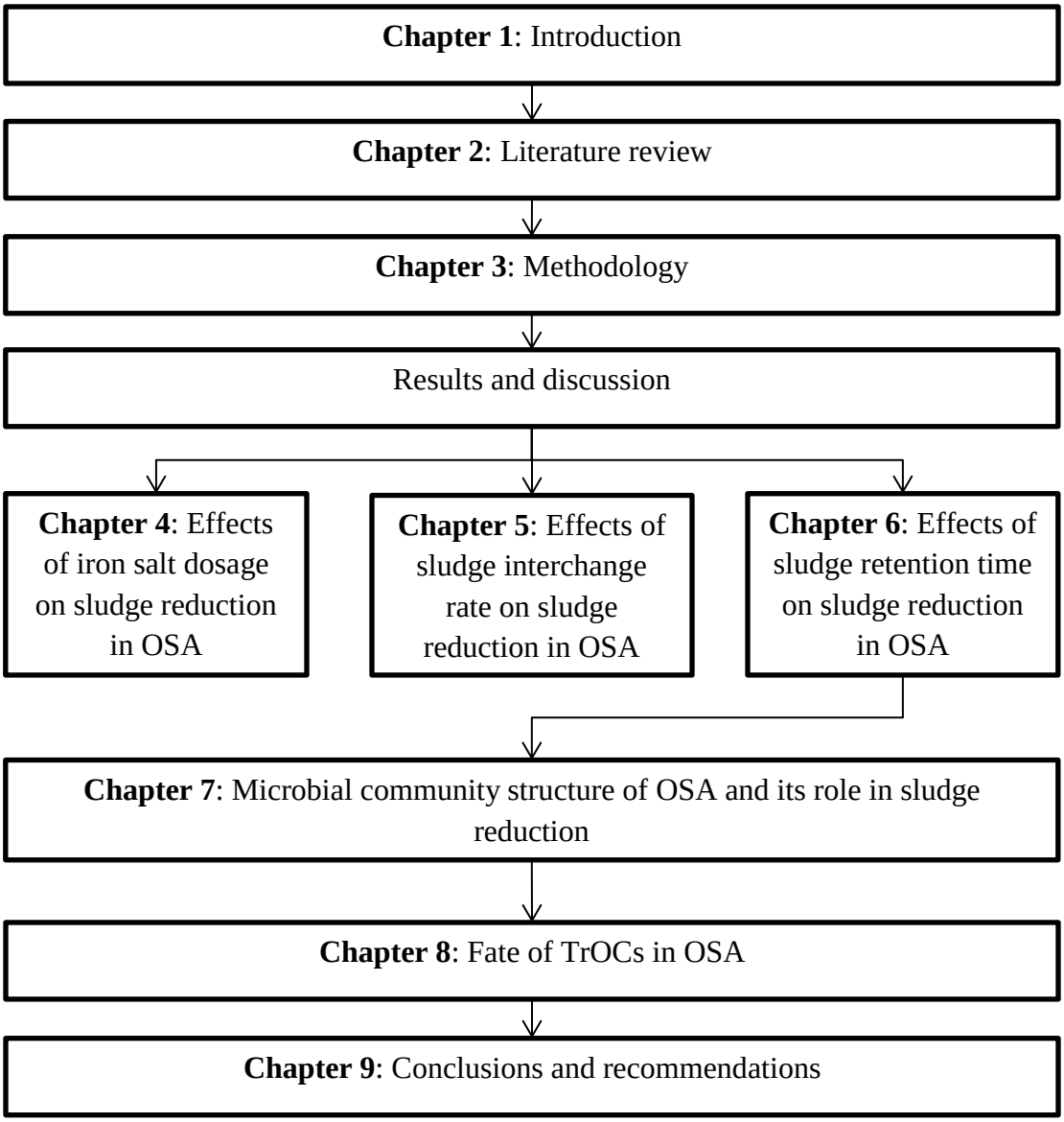

Figure 1.2. Schematic diagram of the thesis outline

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CHAPTER 2: LITERATURE REVIEW

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Semblante, G.U., Hai, F.I., Dionysiou, D.D., Fukushi, K., Price, W.E., Nghiem, L.D. 2016. Holistic sludge management through ozonation: A critical review. *Journal of Environmental Management*, 185, 79-95.

2.1 OVERVIEW OF DIFFERENT APPROACHES TO MINIMISE SLUDGE

Minimising sludge produced by the activated sludge process helps reduce the overall cost of WWTP operation (Guo et al., 2013; Wei et al., 2003). Several innovative approaches were developed to minimise sludge production, and each has its advantages and disadvantages (Table 2.1). The first approach is to control operation parameters such as DO concentration and SRT of the main aeration tank (Table 2.1). Increasing DO concentration enhances the diffusion of oxygen into the sludge flocs and stimulates microbial activity (Wei et al., 2003). Abassi *et al.* (2000) found that increasing DO concentration from 2 to 5 mg/L resulted in 25% sludge reduction in a laboratory-scale CAS. The drawback of increasing DO concentration is the increase in aeration cost. Meanwhile, increasing SRT increases biomass concentration, which results in the decrease of food-to-microorganism (F/M) ratio. This forces microorganisms to expend energy for cellular maintenance rather than propagation (Wei et al., 2003). Increasing SRT decreased sludge production by 12-40% depending on biomass concentration (Low and Chase, 1999). However, it is not possible to adjust SRT to very high levels because it may deteriorate wastewater treatment efficiency and sludge settleability (Wei et al., 2003). Furthermore, increasing SRT also increases oxygen requirements of the aeration tank. Although DO concentration and SRT manipulation is simple and does not require additional chemicals or equipment, it is encumbered by marginal improvement to sludge reduction and additional operation cost (Foladori et al., 2010; Wei et al., 2003).

Table 2.1. Advantages and disadvantages of representative approaches to minimise sludge production during wastewater treatment

Sludge minimisation approach		Advantages	Disadvantages	Selected references
1. Control of operation parameters (DO concentration and SRT)		Easy to implement	High aeration demand; minimal sludge reduction	(Foladori et al., 2010; Wei et al., 2003)
2. Sludge destruction	Thermal treatment	Improves dewaterability; inactivates pathogens	High energy consumption	(Foladori et al., 2010; Neyens and Baeyens, 2003)
	Thermochemical treatment	Improves dewaterability; inactivates pathogens	High energy consumption	(Foladori et al., 2010; Neyens and Baeyens, 2003)
	Ultrasonication	High process	Requires	(Zhang et al.,

		efficiency; improves settleability	expensive equipment; high energy consumption	2007)
	Ozonation	High process efficiency; improves settleability	Requires expensive equipment; high energy consumption; Could form toxic by-products	(Chu et al., 2009; Foladori et al., 2010; Mahmood and Elliott, 2006)
	Chlorination	Less expensive than ozonation	Could form toxic by-products	(Saby et al., 2002)
3. Chemical addition		Easy to implement; does not require additional equipment	Uses potentially toxic chemicals	(Clarke and Smith, 2011b; Foladori et al., 2010)
4. Bacterial predation using protozoa or aquatic worms		Low capital and operation cost; environmentally friendly	Poor process control	(Ghyoot and Verstraete, 2000; Wei et al., 2003)
5. OSA		Low capital and operation cost; environmentally friendly	Less sludge reduction than advanced oxidation processes; process knowledge gaps	(Chen et al., 2003; Foladori et al., 2010; Goel and Noguera, 2006)

The second approach is to destroy RAS before it is re-routed back to the main bioreactor (Table 2.1). Sludge can be destroyed using a number of methods (Table 2.1) including thermal treatment (heating sludge at 40-180^{o}C) (Camacho et al., 2005; Canales et al., 1994; Neyens and Baeyens, 2003), thermochemical treatment (combination of heating and adding acid or base) (Do et al., 2009; Neyens and Baeyens, 2003; Rocher et al., 2001; Uan et al., 2013), ultrasonication (the application of low frequency ultrasonic waves, *e.g.*, 25 kHz or lower) (Vaxelaire et al., 2008; Zhang et al., 2007), ozonation (the application of ozone as oxidizing agent) (Ahn et al., 2002; Kamiya and Hirotsuji, 1998; Yasui et al., 1996), and chlorination (the application of chlorine as oxidizing agent) (Chen et al., 2001; Saby et al., 2002; Takdastan and Eslami, 2013). Sludge destruction results in cell lysis and the release of soluble lysates (products of cell lysis),

which are metabolised by surviving microorganisms. This mechanism is called "lysis-cryptic growth" (discussed in more detail in Section 2.3.1). Methods based on thermal, chemical, or advanced oxidation processes generally have high process efficiency (Foladori et al., 2010). For instance, ozonation achieves up to 100% sludge reduction depending on operation conditions (*e.g.*, ozone dosage, reaction time, and others) and therefore it is currently being applied in pilot- and full-scale plants (Semblante et al., 2016). Moreover, some methods have supplementary benefits such as enhancement of sludge dewaterability and settleability and inactivation of pathogens (Foladori et al., 2010). However, the main disadvantage of this approach is the high capital investment and maintenance cost of the additional equipment (Foladori et al., 2010; Wei et al., 2003). Another disadvantage is evident in advanced oxidation processes, particularly ozonation or chlorination, which can produce toxic by-products that persist in the effluent (Mahmood and Elliott, 2006; Saby et al., 2002).

The third approach is to add chemicals to induce "energy uncoupling" (Table 2.1). Energy uncoupling involves the detachment of catabolism (oxidation of substrate) from anabolism (synthesis of new molecules and cells). This cuts off the energy for cellular propagation and consequently decreases microbial growth (Liu, 2003). This approach is relatively easy to implement and does not require additional equipment (Foladori et al., 2010; Saby et al., 2002). Halogenated phenols (Low and Chase, 1998; Yang et al., 2003) and 3,3',4',5-tetrachlorosalicylanilide (Chen et al., 2002) were found to inhibit microbial growth by interfering with metabolic processes. Nevertheless, phenolic compounds are toxic (Clarke and Smith, 2011b) and 3,3',4',5-tetrachlorosalicylanilide is bioaccumulative, persistent, and toxic to aquatic organisms (USEPA, 2013). Therefore, adding these chemicals activated sludge process could introduce toxicity to either effluent or residual sludge.

The fourth approach is to use bacterial predators such as protozoa and aquatic worms to consume sludge (Table 2.1). The predators feed on bacteria for maintenance, respiration, and reproduction, and their consumption leads to loss of energy and decrease in sludge mass (Ghyoot and Verstraete, 2000). Protozoa was applied in a two-stage process involving a bacteria-rich suspended reactor followed by a protozoa-rich suspended or biofilm reactor. The efficiency of sludge reduction (20-30%) was dependent on the number of protozoa the proliferation of free-floating bacteria that the protozoa are capable of capturing (Ghyoot and Verstraete, 2000; Lee

and Welander, 1996). Various aquatic worms were also applied to consume sludge in a worm-rich reactor, achieving 30-40% sludge reduction (Elissen et al., 2006; Tamis et al., 2011). This approach is potentially low cost and environmentally friendly (Ghyoot and Verstraete, 2000). However, further research is required to understand the impact of operation parameters (*e.g.*, SRT, temperature, and others) on predator growth and to control predator population for continuous reactor operation (Wei et al., 2003).

The fifth approach is to employ the OSA process (Table 2.1), which involves the insertion of external anoxic reactor/s in the RAS loop. Sludge is retained for a certain period in the external anoxic reactor/s wherein it is degraded by biological means. Being a biological approach, OSA is does not require harmful chemicals and is environmentally friendly. Moreover, it has low capital and operation cost relative to other approaches (Foladori et al., 2010; Semblante et al., 2014). Laboratory-scale OSA can reduce sludge by 50-80% (Chon et al., 2011b; Saby et al., 2003; Sun et al., 2010), but full-scale OSA can only achieve up to 20% sludge reduction (Coma et al., 2013; Troiani et al., 2011). Effective transfer and control of OSA technology in full-scale plants will be possible if there were better understanding of the mechanisms responsible for sludge reduction and the factors impacting OSA performance (Foladori et al., 2010; Semblante et al., 2014).

2.2 PERFORMANCE OF OSA AND SIMILAR PROCESSES

Several configurations of OSA and other processes that are fundamentally similar to OSA in terms of design and operation are reported in literature (Coma et al., 2013; Niu et al., 2016; Novak et al., 2007; Saby et al., 2002). These include the generic OSA, anaerobic side-stream reactor (ASSR), Cannibal™, BIMINEX™, and sludge process reduction (SPR) process.

The kinetics of biomass growth is described by the following equation:

$$\frac{1}{Y} = \frac{1}{Y_{max}} + \frac{SRT \cdot k_d}{Y_{max}} \qquad \textbf{Equation 2.1}$$

where Y is the observed sludge yield, Y_{max} is the maximum sludge yield, and K_d is the decay coefficient (Liu and Tay, 2001). The observed sludge yield Y is experimentally determined as:

$$Y = \frac{P}{C} \qquad \textbf{Equation 2.2}$$

where *P* and *C* are the amount of sludge produced and substrate consumed, respectively. *P* is usually expressed as mass (g) of suspended solids and *C* is usually expressed as mass (g) of chemical oxygen demand (COD) (Chon et al., 2011a; Liu and Tay, 2001). In literature, sludge reduction is usually determined by comparing the sludge yield of OSA and a control system that does not have sludge interchange (*e.g.*, OSA *vs.* CAS, MBR-OSA *vs.* MBR, SBR-ASSR vs. SBR, and others) (Novak et al., 2007; Saby et al., 2003) according to the following equation:

$$Sludge\ reduction\ (\%) = \frac{Y_{control} - Y_{OSA}}{Y_{control}} \times 100 \qquad \textbf{Equation 2.3}$$

2.2.1 Generic OSA

The generic OSA involves CAS with an un-aerated external reactor in the RAS loop (Chudoba et al., 1992; Wang et al., 2008; Ye et al., 2008). Some laboratory-scale studies integrated a membrane module in the main aeration tank to form a membrane bioreactor (MBR) (Figure 2.1) that completely retains biomass (An and Chen, 2008; Chen et al., 2003; Saby et al., 2003). The external reactor receives all (An and Chen, 2008; Chen et al., 2003; Saby et al., 2003) or most (Chudoba et al., 1992) of the settled sludge, which contains little substrate due to prior consumption in the main aeration tank (Chen et al., 2003). Because aeration is not conducted in the external reactor, the internal condition is either anoxic or anaerobic (Chudoba et al., 1992; Saby et al., 2003; Ye et al., 2008).

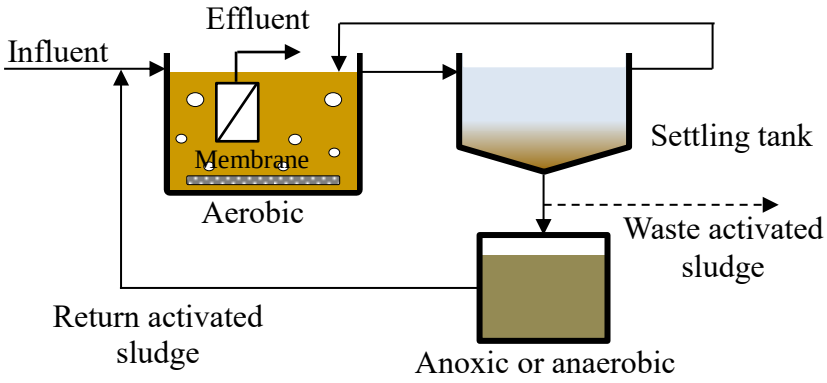

Figure 2.1. Schematic diagram of MBR-OSA

The sludge yield *Y* of laboratory-scale OSA was expressed using various units across different studies (Chen et al., 2003; Chudoba et al., 1992; Saby et al., 2003; Wang et al., 2008). For instance, Chudoba *et al.* (1992) reported that the sludge yield of OSA was 0.20-0.29 g total

suspended solids (TSS)/g COD, whereas Wang *et al.* (2008) reported that the sludge yield was 0.53 g mixed liquor suspended solids (MLSS)/g COD. These corresponded to a sludge reduction of 40-50% (Chudoba et al., 1992) and 13% (Wang et al., 2008) relative to a control CAS (Table 2.2).

Several laboratory-scale studies used MBR-OSA to prevent the run-off of sludge in the effluent and to increase the accuracy of sludge yield measurements (An and Chen, 2008; Chen et al., 2003; Saby et al., 2003). The control system in these studies was an MBR that regularly discarded excess sludge (An and Chen, 2008; Chen et al., 2003; Saby et al., 2003). Saby *et al.* (2003) reported that the sludge yield of MBR-OSA (0.18-0.32 g TSS/g COD) was 20-55% lower than that of the control (MBR 0.40 g TSS/ g COD) (Table 2.2). Meanwhile, Chen *et al.* (2003) observed that the MBR-OSA system had a sludge production rate of 2.3-3.6 g/d, which was 23-51% lower than that of the control MBR (2.39 g/d). An and Chen (2008) reported that the k_d of its anaerobic external reactor (0.13/d) was higher than that of the MBR (0.021/d) and that of typical anaerobic digesters (0.02-0.04/d).

Table 2.2. Summary of the configuration, reactor operation, and performance of OSA and similar processes

Sludge reduction process	Scale	Wastewater	Main reactor for wastewater treatment	External reactor/s for sludge biodegradation	Reactor operation	Control process	Sludge reduction (%)	Reference
Generic OSA	Laboratory	Synthetic	CAS	Anaerobic external reactor	Settled sludge is interchanged with the external reactor	CAS only	13-50	(Chudoba et al., 1992; Wang et al., 2008)
			MBR	Anoxic external reactor		MBR only	20-55	(Chen et al., 2003; Saby et al., 2003)
ASSR	Laboratory	Synthetic	SBR	Strictly anaerobic external reactor	10% of settled sludge is interchanged between SBR and the external reactor (ASSR)	SBR with no sludge wastage	15-45	(Chon et al., 2011a; Novak et al., 2007)
						SBR with aerobic digester	36-40	
						SBR with anaerobic digester	49-54	
Cannibal™	Laboratory	Synthetic	SBR	Strictly anaerobic external reactor	7-10% of sludge is interchanged between SBR and the anaerobic external reactor	SBR only	16-60	(Goel and Noguera, 2006; Novak et al., 2007)

	Full	Real	CAS	"Solids removal module" consisting of physical unit processes (for removal of grits and inert solid) and external anaerobic/anoxic reactor (for biodegradation of sludge)	50% of RAS is interchanged between CAS "solids removal module"	CAS only	Not reported	(Johnson, 2008; Sheridan and Curtis, 2004)
BIMINEX ™	Pilot	Real	UCT process (anaerobic/ anoxic/ aerobic)	Anoxic external reactor	100% of RAS is interchanged between the anaerobic reactor of UCT and the external anoxic reactor	UCT process (anaerobic/ anoxic/ aerobic) only	18	(Coma et al., 2013)
SPR	Laboratory	Real	Anoxic/ aerobic	Micro-aerobic tank and settling tank positioned before the main reactor	The micro-aerobic tank receives settled sludge	Anoxic/ Aerobic only	43-68	(Niu et al., 2016)
			Anaerobic/ anoxic/ aerobic			Anaerobic/ anoxic/ aerobic only	58	(Zhou et al., 2014)

2.2.2 Anaerobic side-stream reactor

A configuration that appears frequently in laboratory-scale studies is a SBR attached to ASSR (Figure 2.2). Chon *et al.* (2011a) and Kim *et al.* (2012) operated an SBR with four cycles per day with especially-timed stages for filling, reaction, settling, and decanting. About 10% of the SBR mixed liquor was allowed to settle, and then transferred to the ASSR once a day. An equal volume of sludge from the ASSR was returned to the SBR at the same time. The SBR-ASSR sludge loop was closed, meaning sludge was only discarded during sampling. Similar to the external reactor of OSA, the ASSR exposed sludge to oxygen- and substrate-deficient conditions to promote biodegradation. However, the ASSR was maintained under strictly anaerobic condition (Chon et al., 2011a; Kim et al., 2012).

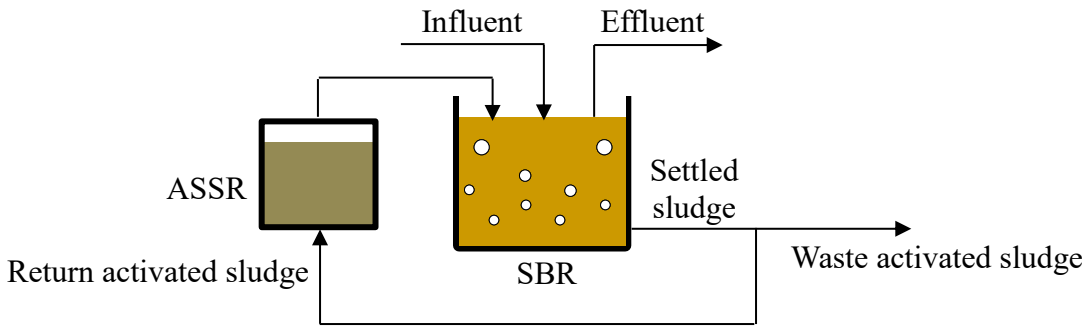

Figure 2.2. Schematic diagram of SBR-ASSR

A few studies investigated sludge reduction in a modified SBR equipped with biological nutrient removal (BNR) attached to ASSR (Datta et al., 2009; Goel and Noguera, 2006). Additional anaerobic and anoxic conditions were achieved during the filling or reaction stages of the SBR through nitrogen purging. This enabled nitrification, denitrification, and orthophosphate uptake/release in the SBR (Datta et al., 2009; Goel and Noguera, 2006).

Independent studies performed by Chon *et al.* (2011a) and Novak *et al.* (2007) both reported a sludge yield of 0.11-0.17 g VSS/g COD for SBR-ASSR. The sludge yield of SBR-ASSR was 15% (Chon et al., 2011a) and 20-45% (Novak et al., 2007) lower than that of a control SBR with no sludge wastage (Table 2.2). Furthermore, it was 36-40% less than that of a control SBR attached to a single-pass aerobic digester (0.27-0.33 g VSS/g COD) and 49-54% less than of another control SBR attached to a single-pass anaerobic digester (0.159 g VSS/g COD) (Chon et al., 2011a).

2.2.3 Cannibal™

The Cannibal® Solids Reduction System by Siemens combines physical and biological approaches to reduce sludge production (Johnson, 2008; Sheridan and Curtis, 2004; Siemens, 2008). It involves the attachment of a "solids removal module" and "interchange reactor" to the main aeration tank. In full-scale Cannibal™, about 50% of RAS is fed to the solids removal module, which contains an intermediate tank, drum screen, and hydrocyclone, for the removal of grit, inert solids, and slowly-biodegradable debris (Figure 2.3) (Johnson, 2008). The output of the solids removal module may have varying solids concentration. The case study of Johnson (2008) has shown that 20-30% of MLSS can be reduced. The collected solids are compressed and discharged. Then, sludge is passed through an anaerobic or anoxic interchange reactor for biodegradation. According to Johnson *et al*. (2008), the interchange reactor was an SBR (SRT=10 d) that returned sludge to the bioreactor. The solids removal module can omitted if the wastewater has minimal amount of inert and slowly-biodegradable solids, *e.g.*, dairy wastewater (Sheridan and Curtis, 2004). Johnson (2008) did not show sludge yield of a full-scale Cannibal™, but Sheridan and Curtis (2004) reported that sludge was reduced to the extent that sludge wastage in the main aeration tank was no longer required (Table 2.2).

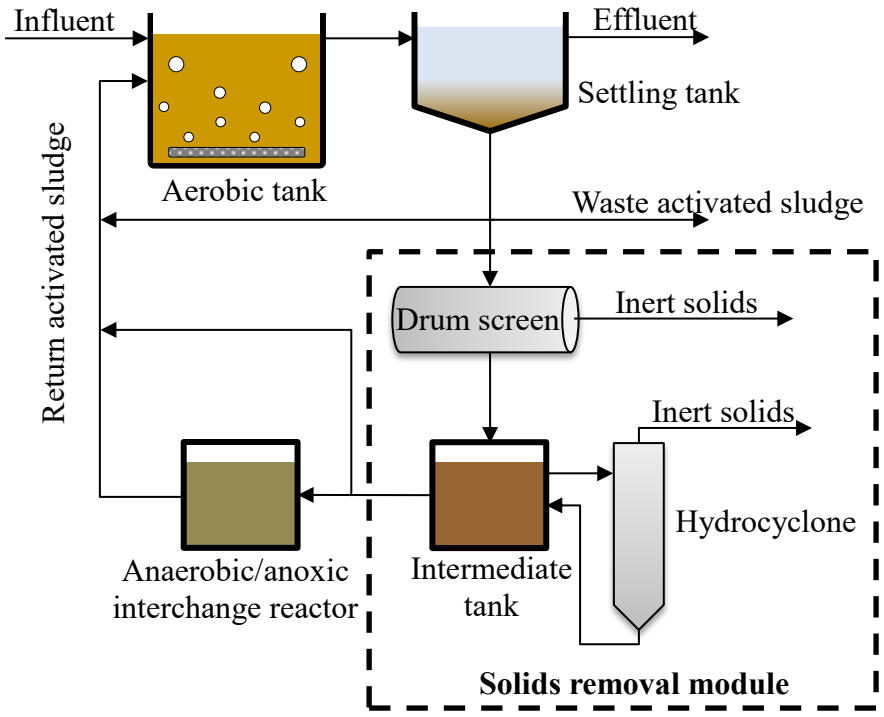

Figure 2.3. Schematic diagram of a full-scale Cannibal™ process

Novak *et al.* (2007) simulated a laboratory-scale Cannibal™ without a solids removal module using an SBR as the main aeration tank. Therefore, their setup was essentially an SBR-ASSR system (Figure 2.2). Settled sludge (50 mL) from the SBR, which represented 4% of the total biomass, was transferred to the anaerobic ASSR (HRT=2 d). Due to extensive sludge accumulation in the main reactor, the amount of sludge that was transferred from the SBR to the ASSR had to be increased to 100 mL (7% of total biomass). The laboratory-scale Cannibal™ achieved 60% sludge reduction relative to a control SBR (Novak et al., 2007).

Goel and Noguera (2006) combined a laboratory-scale SBR with enhanced biological phosphorous removal (EBPR) with an ASSR to study the Cannibal™ mechanism. Similar to the study by Novak *et al.* (2007), Goel and Noguera (2006) did not have a solids removal module and their setup can also be described as an SBR-ASSR (Figure 2.2). The core of the EBPR process in the SBR was the nitrogen-purged anaerobic filling stage followed by aerobic reaction (DO concentration = 5 mg/L) stage. At the end of each cycle, 10% of the settled sludge was transferred to the ASSR. Because sludge was not discarded from the system, the sediment in SBR and the corresponding portion that must be transferred to the

ASSR eventually built up so much that the capacity of the ASSR had to be increased. The configuration achieved 16-33% sludge reduction (Table 2.2).

Even though Novak *et al.* (2007) and Goel and Noguera (2006) aimed to simulate the Cannibal™ process, their setups did not possess the distinctive solids removal module. Moreover, both groups employed synthetic wastewater that contained minimal suspended solids. Domestic and industrial wastewater have varying amounts of inert and slowly-biodegradable debris (*e.g.*, hair and cellulose) that could contribute to sludge volume (Johnson, 2008). Thus, to date, a thorough analysis of the effect of Cannibal's physical pre-treatment on overall sludge reduction has not been made available in the literature.

2.2.4 BIMINEX™

Coma *et al.* (2013) performed simultaneous nutrient removal and sludge reduction by modifying a pilot University of Capetown (UCT) process to include a separate anoxic external reactor, forming the patented BIMINEX™ process (Figure 2.4). The original UCT process employs a succession of anaerobic, anoxic, and aerobic tanks with continuous sludge recycling from anoxic to anaerobic, aerobic to anoxic, and settling to anoxic tanks to enable nutrient removal. In the modified process, the portion of the settled sludge that is customarily returned to the anoxic tank was instead made to pass through the external anoxic reactor for biodegradation, and then returned to the anaerobic tank of the main reactor system. Maintaining anoxic conditions in the external reactor helped ensure that nutrient removal in the main reactor was not interrupted. BIMINEX™ is distinguished from the SBR-ASSRs because of its continuous loading of settled sludge into the SSR (as opposed to intermittent loading). Completely treating the return activated sludge in BIMINEX™ reduced the sludge yield of a full-scale UCT from 0.513 to 0.329 g VSS/g COD, *i.e.*, by 18.3% (Coma et al., 2013).

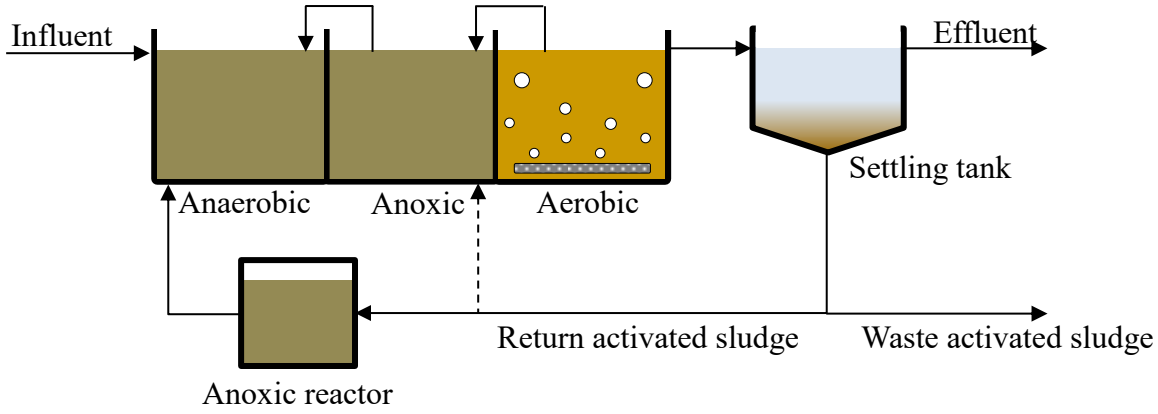

Figure 2.4. Schematic diagram of BIMINEX™

2.2.5 Sludge process reduction

The SPR system involves the addition of an external module, which consists of a micro-aerobic tank and a settling tank, before the main bioreactor (Niu et al., 2016; Zhou et al., 2014). The micro-aerobic tank (0.5-1.0 mg/L) receives both influent and WAS. The micro-aerobic tank is responsible for sludge biodegradation, functioning similarly to the external reactor of OSA. Meanwhile, the additional settling tank provides an anaerobic zone that enhances sludge biodegradation (Niu et al., 2016). This process was implemented in a laboratory-scale study using domestic sewage. It reduced sludge production of a anoxic/aerobic reactor by 43-68% (Niu et al., 2016) and of an anaerobic/anoxic/aerobic reactor by 58% (Zhou et al., 2014) depending on the DO concentration of the micro-aerobic tank.

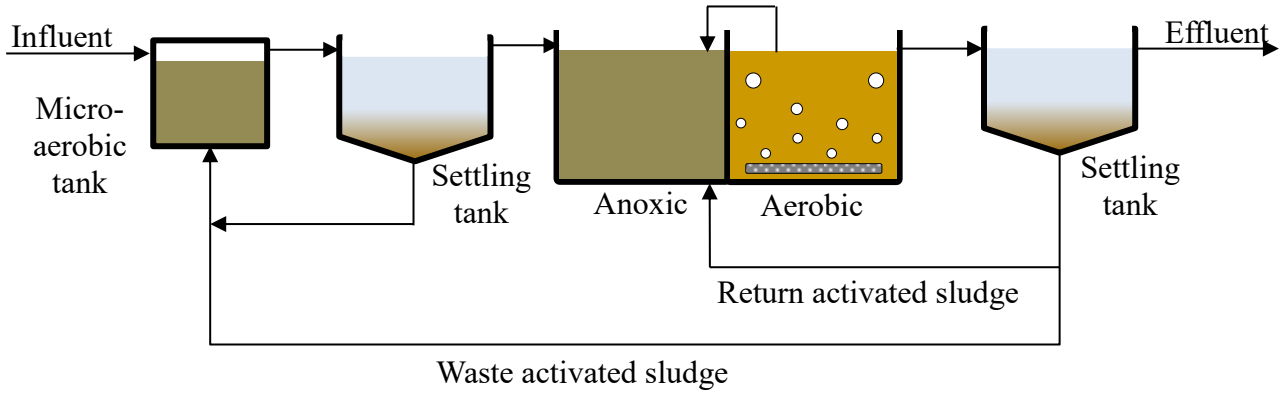

Figure 2.5. Schematic diagram of an anoxic/aerobic activated sludge process with SPR

2.3 POTENTIAL MECHANISMS OF SLUDGE REDUCTION

2.3.1 Lysis-cryptic growth

Generally, biomass growth slows down as external conditions progress from aerobic to anaerobic state. This could be related to the efficiency of energy generation using different electron acceptors. Microbial propagation is most robust under aerobic conditions because substrate oxidation by oxygen gives the maximum amount of free energy. In the absence of oxygen, other compounds such as nitrate, manganese (IV), ferric iron, sulphate, and organic matter can take over as electron acceptor, but less energy is generated (Foladori et al., 2010). The typical maximum sludge yield Y_{max} for aerobic, anoxic, and anaerobic conditions are 0.4, 0.3, and 0.1 g VSS/g COD, respectively (Foladori et al., 2010; Tchobanoglus et al., 2003).

Several studies have shown that the shortage of oxygen and substrate in the external reactors of OSA do not only slow down biomass growth, but also induce in cell lysis (Chen et al., 2003; Chon et al., 2011a; Saby et al., 2003). Cell lysis involves the destruction of the cell membrane and release of lysates (products of cell lysis). It results to endogenous decay, which decreases the activity and mass of sludge (Hao et al., 2010; Liu and Tay, 2001; Wei et al., 2003). Furthermore, under substrate-deficient conditions, surviving microorganisms expend their stored energy exclusively for motility, materials transport, and other activities that would maintain homeostasis (Hao et al., 2010). In other words, they will not use energy for cell propagation (Liu and Tay, 2001; Wei et al., 2003). The same phenomenon is observed other systems with low F/M ratio, such as aeration tanks, MBRs, and digesters that are operated at long SRT, that exhibit lower sludge production compared to CAS (Wei et al., 2003).

Lysates that are released to the supernatant are either biodegradable or non-biodegradable. The biodegradable fraction can be utilised by surviving microorganisms for phosphorus release, sulphate reduction, methane production, and other reactions that do not contribute to biomass growth (Wang et al., 2008). However, when sludge is returned to the main aeration tank, lysates are consumed for cellular propagation in a process called *cryptic growth* (Quan et al., 2012; Wei et al., 2003). A fraction of organic load is lost when lysates are converted to respiration products (*e.g.*, CO_2 and N_2). Therefore, the continuous sludge interchange in OSA causes cell lysis-cryptic growth and a net decrease of biomass (Wei et al., 2003). Although there is strong evidence showing that lysis-cryptic growth is one of the main mechanisms behind OSA (Hao et al., 2010; Liu and Tay, 2001; Wei et al., 2003), it is possible that other mechanisms (*e.g.*, destruction of EPS, discussed in Section 3.5.2) occur simultaneously to

facilitate sludge reduction. Furthermore, the factors that impact lysis-cryptic growth in OSA are not yet identified and optimised.

2.3.2 Destruction of extracellular polymeric compounds

Microbial communities in activated sludge are sustained in complex aggregates or flocs. The formation of sludge flocs is facilitated by extracellular polymeric compounds (EPS), which are proteins, carbohydrates, and other molecules produced by microorganisms. EPS make up the structural framework that is responsible for intercellular adhesion, communication, and propagation. EPS also provide physical protection from bactericides and physical stresses (Liu and Fang, 2003). Some studies suggest that the biodegradation of sludge is impacted by the disintegration of EPS (Ayol et al., 2008; Novak et al., 2003). For instance, Novak *et al.* (2003) found that EPS in the form of proteins and carbohydrates are released to the supernatant when sludge is biodegraded under anaerobic condition. The mechanism through which EPS is solubilised or disintegrated is not yet fully elucidated. However, Ayol *et al.* (2008) suggests that α-amylase and β-glucanase play a role in EPS disintegration and sludge floc destruction (Ayol et al., 2008; Novak et al., 2003).

There is a strong correlation between the concentration of specific cations and volatile suspended solids (VSS) reduction under anaerobic condition (Park et al., 2006). This is because cations function as a "bridge" that connects negatively-charged EPS and microbial cells (Liu and Fang, 2003). It was found that Fe-bound proteins were released when EPS was disintegrated during anaerobic digestion, whereas Ca- and Mg-bound carbohydrates were released during aerobic digestion (Novak et al., 2003; Park et al., 2006). This pattern was observed in OSA-like systems, particularly under anaerobic condition in laboratory-scale Cannibal™ (Novak et al., 2007) and ASSR (Chon et al., 2011a). Chon *et al.* (2011a) stressed that Al- or Fe-bound EPS were especially degraded in the ASSR, suggesting that the concentration of these cations is important in sludge reduction in OSA. Further investigation about the release of EPS in OSA must be undertaken to fully understand the steps involved in EPS disintegration and floc destruction.

2.3.3 Energy uncoupling

Anabolism, or the process of synthesizing new molecules and cells, requires free energy made available by catabolism, or the process of breaking down complex molecules into simpler forms. In aerobic regimes, these metabolic processes are driven by the

phosphorylation and de-phosphorylation of adenosine triphosphate (ATP), which stores large amounts of energy in its covalent bonds. In order to reduce biomass, substrate consumption could be intensified without directing energy towards cellular synthesis. The "uncoupling" of catabolism and anabolism may be induced by addition of certain chemicals (*e.g.*, protonophores, which are compounds that reversibly bind and transfer protons across lipid bilayers) (Liu, 2003), excessive substrate loading (Liu, 1996), and temperature shocks (Foladori et al., 2010). A few studies have systematically investigated the use of protonophores (*e.g.*, chlorophenol and nitrophenol) to reduce sludge (Liu, 2003; Low and Chase, 1999). These compounds disrupt the proton gradient that enables the movement of electrons from substrate to electron acceptor (oxygen). This inhibits the phosphorylation of ATP, and therefore the energy generated from the oxidation of substrate is lost as heat rather than being used for anabolism (Liu, 2003). Some studies hypothesised that energy uncoupling occurs in OSA when sludge is cycled between aerobic and anaerobic conditions. Environmental stress in the external anoxic or anaerobic reactor forces bacteria to expend energy for maintenance metabolism. Sludge reduction occurs when sludge is returned to the aerobic tank, during which they preferentially replenish energy stores. Chudoba *et al.* (1992) demonstrated that ATP concentration decreased after sludge passes through the anaerobic zone. Other systems involving phase cycling (*e.g.*, baffled reactor with three alternating anaerobic and sludge treatment with alternating anoxic and aerobic cycles) have also cited energy uncoupling as means for apparent sludge reduction (Quan et al., 2012; Troiani et al., 2011). Quan *et al*. (2012) operated a sludge-reducing baffled reactor with eight alternating aerobic and anaerobic tanks, and demonstrated that the ATP concentration at the subsequent aerobic tank is less than that of the previous one, indicating the depletion of stored energy in the biomass.

2.4 FACTORS AFFECTING SLUDGE REDUCTION IN OSA

This section critically reviews the available literature on the three factors affecting OSA performance that will be investigated in this study: addition of iron salts, SIR, and SRT. Other factors that are not within the scope of this study (*e.g.*, ORP, temperature, and type of main bioreactor) but have potential to impact sludge reduction are also discussed. Varying the aforementioned factors are not the focus of this study because they may require additional cost or significant changes in reactor design and configuration, which hinders practical full-scale implementation.

2.4.1 Addition of iron salts

The total phosphorous (TP) concentration of domestic sewage usually ranges from 3-20 mg/L. Out of this concentration, 25% and 75% are organic and inorganic phosphorous, respectively. CAS only removes 10-40% of influent TP so large amounts of phosphorous are potentially discharged to receiving water bodies, where eutrophication may occur (Gerardi, 2006; Tchobanoglus et al., 2003). Iron salts (*e.g.*, $FeCl_2$ and $Fe_2(SO_4)_3$) are commonly added directly to the influent or aeration tank in WWTPs for chemical phosphorous removal (Ning et al., 2014; Paul et al., 2001). If Fe(II) salt is added, iron is spontaneously oxidised to Fe(III) given the availability of oxygen in the aeration tank (ORP>+100 mV) (Niu et al., 2013):

$2Fe^{2+} + 2H^+ + ½O_2 \rightarrow 2Fe^{3+} + 2H_2O$, $E°_{cell} = +2.0$ V **Equation 2.4**

Fe(III) reacts with orthophosphate (PO_4^{3-}) according to the basic chemical reaction (Tchobanoglus et al., 2003):

$Fe^{3+} + H_nPO_4^{3-n} \rightarrow FePO_4 + nH^+$ **Equation 2.5**

Fe(III) also forms hydroxyl complexes that serve as "ion bridge" between negatively-charged sites of EPS and causes flocculation (Higgins and Novak, 1997). During flocculation, the outer EPS layer called the "loosely-bound EPS" and the inner EPS layer called the "tightly-bound EPS" are compressed, resulting in the improvement of sludge settleability (Niu et al., 2013). Fe(III) strongly binds with EPS especially those in the form of proteins (Murthy and Novak, 2001). Because of this, Fe(III) decreases the destructibility of flocs. Niu *et al.* (2013) observed that the addition of 5-10 g Fe(III)/g dry solids (DS) prevented the destruction of flocs by shear stress. Similarly, Mishima and Nakajima (2009) found that the addition of Fe(III) at 2-5 g/L to a MBR prevented the release of EPS to the supernatant.

Fe(III) can be reduced to Fe(II) by anaerobic respiration. This causes EPS disintegration and sludge deflocculation (Novak et al., 2003). Park *et al.* (2006) found that anaerobic digestion resulted in EPS solubilisation followed by sludge biodegradation. Similarly, Chon *et al.*, (2011a) reported that the treatment of sludge under anaerobic condition in SBR-ASSR caused EPS solubilisation, and consequently the SBR-ASSR had greater concentration of dissolved EPS than the control SBR. A few studies investigated OSA performance with and without the addition of iron salts to the influent. Novak *et al.* (2007) reported that the sludge reduction of SBR-ASSR was not affected by the addition of 20 m/L of $FeCl_3$ to the influent. Yagci *et al.*

(2015) found increasing the Fe concentration of the influent (2.5 to 16.5 mg/L) increased sludge reduction of OSA (38-78%). However, it is not clear how the addition of iron salts affects OSA performance (Novak et al., 2007; Yagci et al., 2015) especially when other studies have shown that Fe(III) could prevent sludge biodegradation (Mishima and Nakajima, 2009; Niu et al., 2013). Moreover, the disintegration of EPS due to Fe(III) reduction has been demonstrated only under anaerobic condition but not in intermittently aerated (*e.g.*, aerobic/anoxic) regimes that are implemented in OSA. Therefore, a systemaic study is required to determine the impact of iron salt addition on OSA performance.

2.4.2 Sludge interchange rate

SIR is the percentage by volume of sludge interchanged between the main aeration tank or bioreactor system and external reactor/s of OSA. Changing SIR varies the length of time that sludge is exposed to oxygen- and substrate-deficient conditions, and may have important implications on sludge biodegradation in the external reactor/s. However, there is little information in literature regarding the relevance of SIR in OSA operation. The optimum value or range of SIR that facilitates sludge reduction in OSA is also unknown. OSA is usually operated an SIR of 10% (Chon et al., 2011b; Saby et al., 2003; Yagci et al., 2015). Some studies suggest that increasing the fraction of sludge held in the external reactor/s – in other words, increasing SIR – enhances sludge reduction (Coma et al., 2013; Khursheed et al., 2015). Khursheed *et al.* (2015) observed that increasing the ratio of sludge exposed to anaerobic and aerobic conditions (0-8.24 g $MLVSS_{anaerobic}$/g $MLVSS_{aerobic}$) in OSA decreased microbial activity and enhanced sludge reduction (0-39.8%). This suggests that increasing the fraction of sludge or increasing SIR is conducive to sludge reduction. This complements the findings of Coma *et al.* (2013), who reported that increasing the percentage of RAS that is held in the external anoxic reactor of BIMINEX™ increased sludge reduction in the system. On the other hand, Sun *et al.* (2010) increased sludge reduction of SBR-ASSR from 53 to 77% by maintaining SIR at 10% but increasing the frequency of sludge interchange from once per day to four times per day. Given the inconsistent patterns reported in the literature, it is worthwhile to systematically investigate the impact of SIR on OSA performance.

2.4.3 Sludge retention time

Sludge yield is inversely proportional to SRT as depicted by biomass growth kinetics equation (Equation 2.1) (Liu and Tay, 2001; Wei et al., 2003). Increasing SRT increases biomass concentration and decreases F/M ratio, which drives microorganisms to expend energy for cell maintenance rather than propagation (Section 2.1). Up to a certain extent, long SRT can trigger the lysis-cryptic growth mechanism (Section 2.3.1) (Liu and Tay, 2001). Because of this, reactors operated at SRT of infinity (no sludge wastage), *e.g.,* certain MBRs, have lower sludge yield than CAS with fixed SRT (Rosenberger et al., 2000).

In OSA, the addition of external reactor/s essentially prolongs the time spent by sludge in the wastewater treatment line (Saby et al., 2002). This led to the hypothesis that sludge reduction in OSA is driven by the long SRT of the system (Foladori et al., 2010; Semblante et al., 2014). However, studies have shown that OSA systems have lower sludge yield than CAS with longer or infinite SRT. Chon *et al.,* (2011a) reported that the sludge yield of a SBR-ASSR with a total SRT of 74 d (0.11-0.17 g VSS/g COD) was 4-27% lower than that of a control SBR with a SRT of 81 d (0.14-0.186 g VSS/g COD). Novak *et al.* (2007) reported that the sludge yield of SBR-ASSR (0.11 g VSS/g COD) was 20-45% lower than that of an SBR with infinite SRT (0.2 g VSS/g COD). These studies indicate that even though long SRT is beneficial to endogenous decay, it is not the only factor affecting sludge reduction in OSA.

The optimum SRT value or range for OSA is not yet determined. There are contradicting reports on the relationship of SRT and OSA performance. Some studies found that increasing the SRT of the external anoxic reactor of OSA improved sludge reduction (Figure 2.6). Saby *et al,* (2003) observed that sludge reduction achieved by MBR-OSA increased from 23 to 58% when the SRT of the external anoxic reactor was increased from 11 to 17 d (equivalent to increasing the total SRT of the MBR-OSA system from 19.5 to 30.4 d). The authors attributed this to the increase in ORP from +100 to – 250 mV when sludge was retained for a longer period in the external reactor. The shift from aerobic to anoxic condition in the external reactor facilitated the biodegradation of sludge (Saby et al., 2002). Coma *et al.,* (2013) gradually increased the fraction of sludge treated in the external anoxic reactor of BIMINEX™ (0, 10, 50, 100%) and consequently increased the total SRT of the system from 16.5 to 23.3 d. This incrementally enhanced sludge reduction from none to 18%. On the contrary, other studies found that increasing SRT deteriorated OSA performance (Figure 2.6). Chudoba *et al,* (1992) found that sludge reduction of OSA decreased from 50 to 40% when the total SRT of the system was increased from 5 to 12 d. Increasing the SRT reduced the

F/M ratio from 2 to 1 kg COD/kg TSS, which is expected to decrease sludge yield according to classical biomass growth kinetics. However, the authors explained that sludge in OSA has acclimatised to utilise stored energy for maintenance rather than growth, which led to sludge reduction (Chudoba et al., 1992). Ye *et al.* (2008) found that sludge reduction of OSA decreased from 14-33% when the SRT of the external anoxic reactor was increased from 5.5-11.5 h, but the link between SRT and sludge reduction was not clarified. Notably, the range of external reactor SRT that was investigated by Ye *et al.* (2008) (0.2-0.5 d) was significantly lower than that of Saby *et al.* (2003) (11-17 d) so a direct comparison of the two findings is difficult to perform. Thus far, the total SRT of OSA and similar processes reported in literature have been scattered, ranging from very short (*e.g.*, <1 d) to very long (*e.g.*, 80 d) (Figure 2.6). Therefore, it is difficult to establish a correlation between SRT and OSA performance based on the available literature. This is exacerbated by the fact that reports are based on varying wastewater strengths, operation conditions, and methods of quantifying sludge reduction. A systematic investigation of the impact of SRT on sludge reduction will clarify the optimum SRT value or range that will benefit OSA performance.

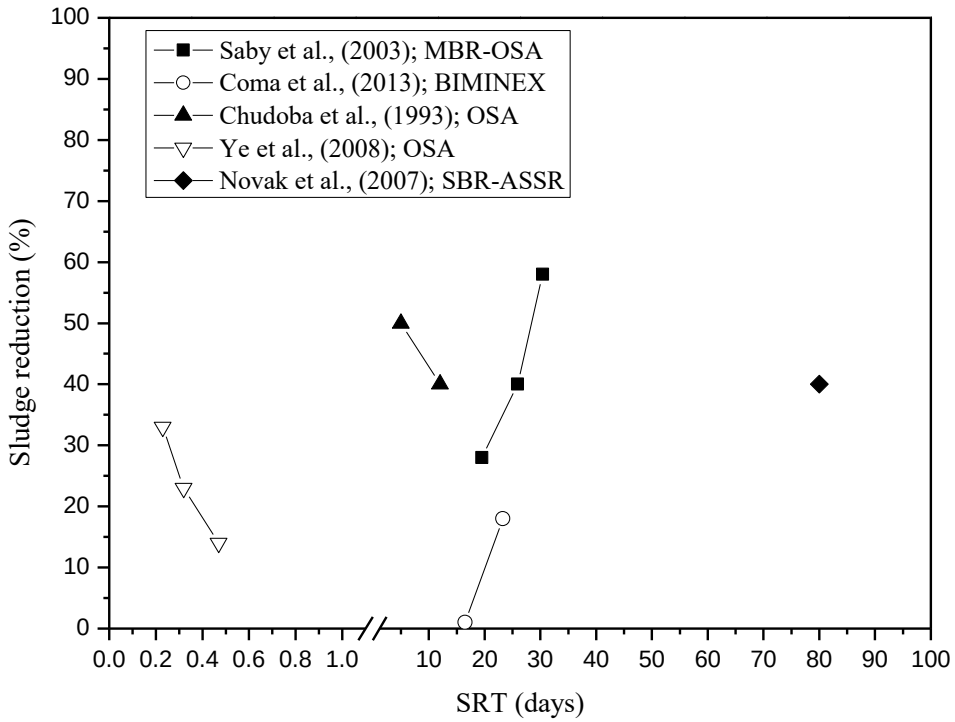

Figure 2.6. Sludge reduction (decrease in sludge yield relative to a control process) achieved by OSA and similar processes operated at various SRT. The impact of SRT on sludge reduction cannot be perceived based on available literature. Data source: (Chudoba et al., 1992; Coma et al., 2013; Novak et al., 2007; Saby et al., 2003; Ye et al., 2008)

2.4.4 Impact of other factors on sludge reduction in OSA

2.4.4.1 Oxidation-reduction potential

ORP measures the net electrical charge of ions in a solution, and reflects the propensity of a solution to gain or lose electrons relative to another solution or substance (Gao et al., 2003). Oxidizing agents such as oxygen increases or makes the ORP value more positive, whereas reducing agents such as organic matter decrease or make ORP more negative. ORP can be used to predict oxidation-reduction reactions in activated sludge. For instance, nitrification occurs in an aerobic reactor with ORP>100 mV (Gao et al., 2003). Denitrification occurs in an anoxic reactor when oxygen is replaced with nitrate as electron acceptor and the ORP value is between +50 and -150 mV (Saby et al., 2003). An anaerobic reactor is realised when oxygen and inorganic nitrogen are unavailable, and it has an ORP level of less than -150 mV (Khanal and Huang, 2003).

Decreasing the ORP of the OSA external reactor decreases sludge yield. Saby e*t al.* (2003) observed that decreasing the ORP values of the external reactor in an MBR-OSA from +100 to -250 mV decreased sludge yield from 0.32 to 0.18 g MLSS/g COD. In other words, it is necessary to maintain oxygen-deficiency in the external reactor of OSA to enable sludge reduction. It is difficult to assess the effect of ORP on sludge yield across different studies because the reactor configuration, SRT, and units of sludge yield differed (Table 2.3). Nonetheless, regardless of these variations, it appears that an ORP -250 mV consistently resulted in low sludge yield (Table 2.3).

Table 2.3. Sludge yield at different ORP and SRT of OSA and similar processes

Sludge reduction process	ORP (mV)	SRT (d)	Y_{obs}	Reference
OSA	-250	5	0.20 [a]	(Chudoba et al., 1992)
OSA	-250	12	0.29 [a]	(Chudoba et al., 1992)
OSA	-250	Not reported	0.49 [b]	(Wang et al., 2008)
MBR-OSA	-250	30	0.18 [b]	(Saby et al., 2003)
BIMINEX	-150	26	0.33 [c]	(Coma et al., 2013)
MBR-OSA	-100	26	0.22 [b]	(Saby et al., 2003)

MBR-OSA	+100	19.5	0.32 [b]	(Saby et al., 2003)

[a] g TSS/g COD
[b] g MLSS/g COD
[c] g VSS/g COD

Strict ORP control could be a costly and impractical exercise. For instance, the ORP of sludge is adjusted in laboratory-scale studies through the injection of nitrogen of gas (An and Chen, 2008; Saby et al., 2003). Troiani *et al.* (2011) pointed out that it is difficult to maintain the ORP at a certain value. They operated a full-scale bioreactor with alternating redox conditions in the sludge line. Two ranges of ORP were applied, *e.g.*, -400 to -200 mV (to favour facultative anaerobic biomass) and -200 to +50 mV (to favour facultative aerobic biomass). Interestingly, they found that maintaining these ORP ranges for equal duration resulted to the least growth yield of 0.09 g VSS/g COD. This could have two important implications for the operation of OSA or similar configurations. First, an ORP range sufficiently activates sludge reduction activities (as opposed to a strict ORP). Second, alternating anoxic and anaerobic stages in OSA or SSR could be more beneficial than maintaining either stage alone.

2.4.4.2 Temperature

Laboratory-scale OSA and similar processes were usually operated at 20-25^{o} C (Chen et al., 2003; Chon et al., 2011a; Chudoba et al., 1992; Goel and Noguera, 2006; Novak et al., 2007; Wang et al., 2008; Ye et al., 2008), whereas full-scale systems were operated at ambient temperature (Coma et al., 2013; Troiani et al., 2011). However, increasing temperature could enhance sludge reduction (Foladori et al., 2010; Yang et al., 2011). For instance, floc destruction in thermophilic aerobic and anaerobic digesters is attributed to kinetic acceleration of biochemical reactions and selection of thermophilic bacteria that could induce enzymatic hydrolysis of cell walls (Foladori et al., 2010). Yang *et al.* (2011) used surface response methodology to model sludge reduction in alternating aerobic/oxygen-limited environment over the range of 20-30^{o} C, and found that the ideal temperature is 29^{o} C. While high temperature supports uncoupled metabolism (Foladori et al., 2010), extreme heat could impact biological activity and sludge properties (Tchobanoglus et al., 2003). It should be noted that 29 ^{o}C is slightly higher than what is usually adopted in OSA as described in literature. Although it is interesting to investigate the effect of temperature on sludge

reduction, increasing the temperature will require additional equipment and energy consumption that will ultimately increase the operation cost of WWTPs.

2.4.4.3 Type of main bioreactor

Most laboratory-scale studies employed an aerobic tank as the main bioreactor of OSA (Chen et al., 2003; Chon et al., 2011a; Chudoba et al., 1992; Novak et al., 2007; Wang et al., 2008; Ye et al., 2008). There is little information on effect of applying OSA to more complex main bioreactors such as those that have integrated anaerobic, anoxic, and aerobic compartments for nutrient removal. Goel and Noguera (2006) attached an ASSR to a SBR with anaerobic and aerobic reaction stages and achieved 16% sludge reduction. Datta *et al.* (2009) attached an ASSR (without sludge wastage) to a SBR with anaerobic, anoxic, and aerobic stages and compared its sludge production with a control SBR attached to an anaerobic digester (with sludge wastage). The SBR-ASSR achieved 63% sludge reduction relative to the control SBR-anaerobic digester. Datta *et al.* (2009) pointed out that greater sludge reduction occurred when the SBR was operated at anaerobic/aerobic/anoxic/aerobic mode than anaerobic/aerobic/anoxic mode. The authors suggested that the state of the sludge that enters the ASSR was relevant to the sludge reduction process. This finding was supported by Novak *et al.* (2007), who hypothesised the movement of recycled biomass from aerobic to anaerobic conditions is key to the release of Fe and solubilisation of organic matter. Therefore, transferring aerated biomass to the ASSR caused additional sludge destruction than transferring anoxic biomass. However, no further data was provided to support this hypothesis.

SPR process was integrated to main bioreactors with anaerobic/anoxic (Niu et al., 2016) and anaerobic/anoxic/aerobic tanks (Zhou et al., 2014). The sludge reduction achieved in these studies were high (43-68%), which further shows evidence that OSA and similar process can be implemented using any type of main bioreactor. Nonetheless, it is possible for the anaerobic and anoxic environments in the main bioreactor to trigger sludge reduction mechanisms that are different from those that occur at strictly aerobic condition. Further study is necessary to elucidate and apply such mechanisms in full-scale WWTPs.

2.5 MICROBIAL COMMUNITY STRUCTURE OF OSA

The microbial community structure of natural or man-made biological systems, such as that of activated sludge, have significant impact on microbial activity, biomass properties, and

process efficiency (Da-Zhi et al., 2016; Ferrera and Sánchez, 2016). The unique system configuration of OSA (*i.e.*, sludge interchange between conditions that are rich and deficient in oxygen and substrate) may enable it to have a different microbial community structure from CAS. DO concentration is a major factor affecting microbial diversity (*i.e.*, the variability of species) of activated sludge (Ning et al., 2014; Stadler and Love, In press; Yadav et al., 2014) and other biological matrices (*e.g.*, marine estuaries) (Spietz et al., 2015). Generally, microbial diversity increases as the DO concentration decreases due to the emergence of facultative anaerobes and other bacteria that can thrive in the absence of oxygen (Spietz et al., 2015; Yadav et al., 2014). Moreover, the availability of substrate affects the activity and population of different bacteria. Starvation of biomass can stimulate the growth of one bacterial group while causing the decline of others (Pijuan et al., 2009; Xing et al., 2016). Therefore, the oxygen- and substrate-deficient zones in OSA have high potential to cause variation in microbial diversity and composition that are possibly different from that of conventional systems. Determining the distinctive microbial community structure of OSA can deliver valuable insights on sludge reduction mechanisms. It can also broaden the current understanding of bioreactors with analogous environmental conditions (*e.g.*, anaerobic digesters).

Some studies have confirmed that the microbial community of OSA (main aeration tank and external reactor/s) is different from that of CAS (Goel and Noguera, 2006; Kim et al., 2012; Wang et al., 2008). Wang *et al.* (2008) used PCR-DGGE analysis and observed that OSA had greater microbial diversity than CAS. Kim *et al.* (2012) also employed PCR-DGGE analysis and found that the microbial profile of a SBR attached to ASSR was similar to CAS. On the other hand, the microbial profile of ASSR was similar to that of an anaerobic digester. The ASSR harboured bacteria associated with nitrite-to-nitrate conversion, EPS formation, and phosphate release. The dominant phyla that were detected in the ASSR were *Proteoebacteria*, *Spirochaetes*, *Clostridiales*, *Chloroflexi*, and *Actinobacteria*. Furthermore, oxygen-deficiency in OSA enriched slow-growing bacteria such as fermenters and polyphosphate-accumulating organisms (PAOs) in both the main aeration tank and external reactor/s (Chudoba et al., 1992; Goel and Noguera, 2006). Goel and Noguera (2006) detected fermentative bacteria in a SBR attached to ASSR but not in a control SBR. Because fermentative bacteria were able to break down slowly-biodegradable substrates, the COD removal efficiency of the SBR-ASSR was greater than that of the control SBR. Goel and Noguera (2006) also observed significant PAO activity (*i.e.*, the release of orthophosphate in the aerobic phase) in a SBR-

ASSR but not in the control SBR. Similarly, Chudoba *et al.* (1992) observed that the number of PAOs in the main aeration tank of OSA (50-60% of the population) greater than that of the control CAS (5-10%). Meanwhile, Quan *et al.*, (2012) detected hydrolytic-fermentative bacteria and acetogenic bacteria in the anaerobic compartments of a baffled reactor with repeated sequences of aerobic and anaerobic conditions. Clearly there is convincing evidence showing that OSA has greater microbial diversity (Kim et al., 2012; Wang et al., 2008) and population of slow-growing bacteria (Chudoba et al., 1992; Goel and Noguera, 2006) than CAS. However, the implications of the aforementioned patterns on sludge reduction are not fully understood. Notably, the aforementioned findings are based on low-throughput techniques (*e.g.*, PCR-DGGE and measurement of bacterial activity). Having low sensitivy and accuracy, these techniques provide inadequate information on microbial diversity and taxonomic classification. Therefore, they are unable to fully characterise the microbial community structure of dynamic biological systems.

Recently, high-throughput techniques such as 454 pyrosequencing (Ning et al., 2014; Zhou et al., 2015; Zhou et al., 2014) and Illumina sequencing (Niu et al., 2016) were applied to study OSA. These techniques have high sensitivity and can identify bacterial populations down to the phylum, class, and order level. Ning *et al.* (2014) operated an OSA system consisting of an anoxic/aerobic main reactor attached to an external anaerobic reactor. Using 454 pyrosequencing, they observed that phyla *Proteobacteria* and *Bacteroidetes* were abundant in OSA and control CAS, but *Proteobacteria* was lower in OSA than CAS. They further identified that class *β-Proteobacteria* was significantly reduced in OSA, whereas class *Spingobacteria* (fermentative bacteria) was higher in OSA than CAS. The authors hypothesised that *Sphingobacteria* may play a key role in sludge reduction (Ning et al., 2014). Zhou *et al.* (2015) operated anoxic/aerobic main reactor attached to an external anoxic reactor and used 454 pyrosequencing to show that fermentative acidogenic bacteria (classes *Anaerolineae* and *Actinobacteria*) uniquely emerged in the external anoxic reactor possibly due to hydrolytic decay. Meanwhile, Zhou *et al.* (2014) operated an anoxic/aerobic main reactor with a preceding SPR module *(Section 2.2.5). They found via 454 pyrosequencing that* fermentative bacteria belonging to the class *Anaerolineae, Actinobacteria, Cytophagia* and *Caldilineae and the predatory bacteria Myxobacteria were enriched the micro-aerobic tank of the SPR module. Overall, the microbial diversity of the anoxic/aerobic-SPR system was greater than that of the control anoxic/aerobic system* (Zhou et al., 2014). Niu et al. (2016) operated an anoxic/aerobic main reactor with a preceding SPR module *(Section 2.2.5)*

and varied the DO concentration the micro-aerobic tank. Illumina sequencing revealed that decreasing the DO concentration of the micro-aerobic tank from 2.5 to 0.5 mg/L increased its microbial diversity. Fermentative (class Spingobacteria and Anaerolineae) and predatory bacteria (class Bdellovibrio and Bacteriovorax) were especially enhanced at low DO concentrations (Niu et al., 2016). *Generally, findings based on high throughput techniques reveal that although OSA causes cell lysis under environmental stress, specialised bacterial groups (fermenters, hydrolyzers, and predators) flourish probably due to their unique ability to adapt to oxygen- and substrate-deficient conditions* (Ning et al., 2014; Niu et al., 2016; Zhou et al., 2015; Zhou et al., 2014).

Although significant progress has been made using high-throughput techniques, further research is still required to relate the microbial community structure to OSA mechanisms and performance. For example, it will be worthwhile to examine the changes in microbial diversity and population density of bacterial groups in response to varying operation conditions. This will help address the knowledge gaps and facilitate the design and optimisation of OSA configurations.

2.6 IMPACT OF OSA ON BIOLOGICAL WASTEWATER TREATMENT

2.6.1 COD removal

It is imperative that applying sludge reduction processes in WWTPs do not interfere with the primary objective of biological wastewater treatment, *i.e.,* to remove organic matter, nutrients, and other pollutants from the influent. The biodegradation of sludge in the external reactor/s of OSA increases soluble COD due to the release of lysates. Therefore, returning biodegraded sludge to the main aeration tank has potential to increase the COD of the effluent. Nonetheless, several studies report that OSA did not have negative impact on the COD removal efficiency of the main bioreactor (Table 2.4). Saby *et al.* (2003) observed that the surplus soluble COD generated in the external anoxic reactor of MBR-OSA was rapidly consumed when sludge was returned to the MBR. Goel and Noguera (2006) reported the COD removal efficiency of SBR-ASSR (98%) was slightly higher than a control SBR (97%), and explained that the enhancement was due to the conversion of COD to polyhydroxyalkanoate (PHA) by PAOs that were enriched in the SBR-ASSR system. An and Chen (2008) demonstrated that the surplus COD produced in OSA may benefit several bacterial activities. They simulated anoxic and anaerobic OSA in closed batch reactors by

intermittently purging nitrogen gas and completely withholding nitrogen and oxygen gases, respectively. Soluble COD increased in both reactors due to sludge biodegradation, but there was a discrepancy between the actual and expected soluble COD concentrations. Mass balance analysis showed that soluble COD was consumed consumed for denitrification, sulphate reduction, phosphorous release, and methane production.

Table 2.4. Effect of OSA and SSR on wastewater treatment efficiency (COD, phosphorous, and nitrogen removal) and sludge settleability

Sludge reduction process	**Control process**	**Sludge reduction (%)**	**COD removal (%)**		**Nitrogen removal (%)**		**Phosphorous removal (%)**		**SVI (mL/g)**		**Reference**
			Sludge reduction process	*Control process*	*Sludge reduction process*	*Control process*	*Sludge reduction process*	*Control process*	*Sludge reduction process*	*Control process*	
OSA	CAS	50	82-99	83-95	-	-	19-42[d]	1-16[d]	250-1000	740-1900	(Chudoba et al., 1992)
OSA	CAS	14-33	93	90-91	30[a]	28-30[a]	48.9[c]	48-58[c]	60	65-90	(Ye et al., 2008)
MBR-OSA	MBR	23-51	91	91	-	-	-	-	-	-	(Chen et al., 2003)
MBR-OSA	MBR	20-55	92	91	-	-	28-63[c]	64 [c]	$ORP_{-100\ mV}$ = 90-290 $ORP_{-250\ mV}$ = 50-100	$ORP_{-100\ mV}$ = 175-300 $ORP_{-250\ mV}$ = 250-300	(Saby et al., 2003)
SBR- ASSR	BNR SBR	63	-	-	-	-	90[d]	90-95[d]	-	-	(Datta et al., 2009)
SBR- SSR	EBPR SBR	16-33	98	97	100[b]	100[b]	97[d]	84 [d]	-	-	(Goel and Noguera, 2006)

[a]Percentage based on TN removal
[b]Percentage based on NH_3-N removal
[c]Percentage based on TP removal
[d]Percentage based on PO_4^{3-}-P removal

2.6.2 Nitrogen removal

There are only a few reports on the effect of OSA on the nitrogen removal efficiency of the main bioreactor, but the available studies suggest that OSA does not have negative impact (Table 2.4). For instance, Ye *et al.* (2008) reported that the total nitrogen (TN) removal efficiency of CAS (30%) was similar to that of the control CAS (28-30%). Similarly, Datta *et al.* (2009) observed that the effluent ammonium, nitrate, and nitrite concentration of SBR-ASSR and control SBR were similar. Cycling sludge between the main aeration tank and external anoxic or anaerobic reactor have potential to create a condition that enables BNR (enhanced nitrification and denitrification) especially when surplus COD is produced from sludge biodegradation. Saby *et al.* (2003) showed that the effluent nitrate concentration of MBR-OSA (11-25 mg/L) was lower than that of the control MBR (34 mg/L). Hence, denitrification in MBR-OSA was potentially more robust the control MBR. Further study is necessary to confirm the effect of OSA on nitrification and denitrification efficiency of main bioreactors.

2.6.3 Phosphorous removal

There are contradicting reports on the effect of OSA on phosphorous removal of the main bioreactor (Table 2.4). Some studies observed that OSA improved phosphorous removal. For example, Chudoba *et al.* (1992) showed that the orthophosphate removal efficiency of OSA (19-42%) was higher than that of the control CAS (2-18%). This was probably because OSA had a significantly greater population of PAOs (60% of the total bacterial community) than the control CAS (10%). Similarly, Ye *at al.* (2008) found that the TP removal efficiency of OSA (28-30%) was slightly higher than that of the control CAS (48.9%). They hypothesised that the enhancement was due to higher substrate loading and the adsorption of phosphorous on biomass. Goel and Noguera (2006) reported that the effluent PO_4^{3-} concentration of EBPR SBR-ASSR (0.3 mg/L) was comparable with the control EBPR SBR (1.5 mg/L). On the contrary, Saby *et al.* (2003) observed that orthophosphate was released in the external anoxic reactor of OSA especially at low ORP (-250 mV). Therefore, the effluent orthophosphate concentration of MBR-OSA increased from 3.7 to 7.2 mg/L when ORP of the external reactor was decreased from +100 to –250 mV. Notably, the aforementioned values were below the orthophosphate discharge standards. Goel and Noguera (2006) also observed that orthophosphate concentration increased in the ASSR, but the surplus orthophosphate was taken up by PAOs when sludge was returned to the anaerobic stage of the main bioreactor

(SBR). These studies imply that although sludge biodegradation has potential to release orthophosphate, the quality of the effluent can be maintained if the increase in orthophosphate is low or appropriate control strategies are implemented (*e.g.*, EBPR).

2.6.4 Sludge settleability

The settleability of sludge is crucial in CAS because it determines the separation efficiency of the effluent from biomass (Tchobanoglus et al., 2003). "Sludge bulking" or the failure of sludge to settle occurs due to the proliferation (1-20% volume fraction) of filamentous bacteria in the bioreactor (Martins et al., 2004). The growth of filamentous bacteria is affected by ammonia concentration, DO concentration, temperature, and other environmental factors. Filamentous bacteria possess a long thread-like morphology with large surface area, and thus settle more slowly than normal floc-forming bacteria (Rossetti et al., 2005). These microorganisms has also been associated with foaming or the excessive formation of gas bubbles on the surface of bioreactors or settling tanks (Gardoni et al., 2011).

Some studies reported that OSA improved sludge settleability (Table 2.4). High sludge volume index (SVI), *i.e.*, the volume in mL occupied by 1 g of activated sludge after 30 min of settling, implies poor sludge settleability due to bulking (Liu and Fang, 2003). A few studies have observed that OSA had lower SVI (*i.e.*, better sludge settleability) compared to CAS (Chudoba et al., 1992; Ye et al., 2008). Ye *et al.* (2008) noted that SVI affected by the SRT of the external anoxic reactor (5.5-11.5 h) of OSA. The SRT of 7.6 h resulted in the most stable SVI readings. The dependence of SVI on SRT may be explained by the fact that various filamentous bacteria grow at different SRT, but this was not verified in the study because bacterial characterization as not performed (Ye et al., 2008). Saby et al. (2003) also observed that the SVI of the MBR-OSA was lower than that of a control MBR . They speculated that the EPS released by sludge biodegradation functioned as flocculant that helped improve sludge settleability.

2.6.5 Sludge dewaterability

Sludge dewatering is a downstream process used to decrease the moisture content and volume of sludge. Removing water from sludge is necessary to minimise the cost of sludge handling and transportation, to facilitate other sludge downstream processes (*e.g.*, incineration), and to meet standards for the land application of biosolids. The process is constrained by colloidal particles and EPS, which have high affinity towards water molecules

(Mowla et al., 2013; Park et al., 2008; Tchobanoglus et al., 2003). It was observed that the disintegration of EPS by thermal treatment or oxidative treatment reduces the water retention of sludge (Neyens et al., 2004). Anaerobic digestion of sludge partially disintegrates EPS, but alters EPS composition such that it contains more proteins than polysaccharides. The result is the deterioration of sludge dewaterability (Houghton et al., 2000). Based on the patterns observed in anaerobic digestion, it is possible for sludge cycling (*e.g.,* aerobic/anoxic) in OSA to affect sludge dewaterability. Further study is needed to determine the dewatering properties of OSA sludge especially at varying operation conditions.

2.7 FATE OF TrOCs IN OSA

TrOCs are pesticides, industrial chemicals, components of consumer products, pharmaceuticals and personal care products, hormones, and other organic pollutants that are ubiquitous in domestic sewage and other environmental matrices. Many of these contaminants have potential to disrupt the endocrine system and cause developmental abnormalities in animals and humans (Citulski and Farahbakhsh, 2010). There is significant concern over the fate of TrOCs in WWTPs, which may serve as point sources for further recirculation of the contaminants in water bodies and soils (Birkett and Lester, 2003; Citulski and Farahbakhsh, 2010; Clarke and Smith, 2011a). There is a wealth of knowledge on the fate of TrOCs in CAS and sludge handling or treatment units (*e.g.,* anaerobic digestion), but their fate in OSA and similar processes is unknown. This section reviews the literature on the fate of TrOCs during biological wastewater and sludge treatment, which may help shed light on the potential sorption patterns and biodegradation pathways of TrOCs in OSA.

2.7.1 Fate of TrOCs in biological wastewater treatment

TrOCs may sorb on sludge flocs, undergo biodegradation or abiotic transformation, or remain unchanged. TrOC sorption on sludge largely depends on their physico-chemical properties, *e.g.,* hydrophobic TrOCs are more likely to partition in the organic portion of sludge (to be discussed in Section 2.7.1). Although sorption separates TrOCs from wastewater, it does not result to their elimination from the sludge and therefore not a means of "TrOC removal." TrOC biodegradation mostly occurs via co-metabolic pathways, and may result in either the complete mineralisation of the contaminants or the formation of metabolites (to be discussed in Section 2.7.1.2.). Generally, abiotic loss of TrOC in primary or secondary wastewater treatment and in sludge treatment is minimal (to be discussed in Section 2.7.1.3).

2.7.1.1 TrOC sorption

Activated sludge has a high sorption capacity for TrOCs due to its large specific surface area (Birkett and Lester, 2003). Sorption occurs mostly through hydrophobic interactions between TrOCs and the organic fraction of sludge (Li et al., 2013; Zhang et al., 2012). Li *et al.* (2013) reported a positive correlation between the sorption of antibiotics and the total organic carbon (TOC) of secondary sludges from different WWTPs (e.g. 7-45% TOC). Similarly, Zhang *et al.* (2012) observed that the sorption of 17α-ethinylestradiol increased with the TOC of different types of sludge (e.g. 44-47% TOC). A direct relationship between TrOC sorption and TOC is also observed in other environmental matrices, such as soils (e.g. 5-33% TOC) and aquatic sediments (e.g. <1-5% TOC) (Zhou et al., 2011).

Sorption increases with hydrophobicity of TrOCs, which can be quantified using the apparent partition coefficient *log D*:

$$\log D = \frac{[HX]_o}{[HX]_w + [X^-]_w} \qquad \textbf{Equation 2.6}$$

where $[HX]_o$ is the concentration of the un-ionised form of the compound partitioned in octanol and $[HX]_w$ and $[X^-]_w$ are the concentrations of the un-ionised and ionised forms of the compound partitioned in water, respectively, when the octanol-water system is under equilibrium at a given pH and temperature. According to Hai *et al.* (Hai et al., 2014), TrOCs with log D > 3 generally have high sorption on sludge. Hydrophobicity depends on the chemical structure of the compound. For example, Niu *et al.* (2013) observed that the sorption of perfluorosulphonate on sludge is significantly higher than that of perfluorocarboxylate because the sulphonate group is more hydrophobic than the carboxylate group. Nonetheless, Taedkaw *et al.* (2011) found that hydrophobic TrOCs that possess electron donating groups (*e.g.*, hydroxyl and amine) do not accumulate in the sludge of an aerobic membrane bioreactor (MBR) due to their high biodegradability.

TrOCs also sorb on sludge by means of electrostatic attraction. The pH at the isoelectric point (pI) of sludge is 2.9 (Wang et al., 2000), meaning its surface is negatively-charged under typical biological conditions of pH 7. Therefore, TrOCs that predominantly exist in their neutral or positively-charged forms at pH 7 have high sorption in primary and secondary sludge (Hyland et al., 2012; Lindberg et al., 2005; Stevens-Garmon et al., 2011). On the other hand, TrOCs that are predominantly negatively-charged at pH 7 did not significantly sorb on

sludge due to electrostatic repulsion (Gao et al., 2012). Favourable electrostatic conditions also facilitate hydrophobic interactions, as noted by Urase and Kikuta (2005) when they found that a linear correlation between hydrophobicity and sorption existed only when TrOCs are predominantly in neutral form. However, exceptions have been reported in literature. Calace *et al.* (2002) observed that although electrostatic repulsion is expected between negatively-charged chlorophenols and sludge at pH 8, high sorption of the compounds still occurred via hydrophobic binding. Similarly, Stevens-Garmon *et al.* (2011) found that some positively-charged compounds (*e.g.,* trimethoprim and atenolol) that are expected to have high electrostatic attraction with sludge at pH 7 exhibited low sorption due to their hydrophilic nature.

Extracellular polymeric products (EPS) may play a significant role in TrOC sorption. EPS are highly hydrophobic, and therefore have higher affinity towards organic pollutants (*e.g.* benzene and toluene) than cell walls of microorganisms (Sheng et al., 2010). Thus far, Niu *et al.* (2013) observed a positive correlation between the sorption of perfluoroalkyls and the protein fraction of EPS, and attributed their binding to the linkage of the compounds with the amide group or secondary structure of protein. Likewise, Métivier *et al.* (2013) found that the erythromycin has greater affinity towards EPS than acetaminophen, which may explain why erythromycin has greater sorption on sludge. Khunjar and Love (Khunjar and Love, 2011) performed TrOC sorption experiments on sludge with and without EPS (*e.g.*, attained by cation exchange resin extraction). They observed that 17α-ethinylestradiol have greater affinity towards the protein fraction for EPS, whereas trimethoprim sorbed equally on the protein and polysaccharide fractions. Further investigation is required to confirm the relationship of EPS and TrOC sorption, but this is probably challenging because EPS characteristics are sensitive to many factors including wastewater characteristics, bacterial growth phase, and reactor operation conditions (Sheng et al., 2010).

Notably, the irreversible sorption of organic contaminants and their metabolites has been observed in soils (Gevao et al., 2000). There is limited information on the irreversible sorption of TrOCs on activated sludge, but it is likely to have impact on TrOC bioavailability and treatment. It may also result to non-extractable residues (*i.e.,* compounds that cannot be liberated from the sludge flocs without significantly altering the sludge matrix) and obfuscate sample extraction an analysis (Boxall et al., 2012; Brooks et al., 2009). The eventual liberation of these irreversibly bound TrOCs under specific conditions, *e.g.* when volatile solids are destroyed or sludge is exposed to soil (Kouloumbos et al., 2008; Lillenberg et al.,

2010), is also of environmental concern (Boxall et al., 2012). The liberation of TrOCs upon destruction of volatile solids has been observed during anaerobic digestion (Section 2.7.3.2)

2.7.1.2 TrOC biodegradation

Due to their low concentration of TrOCs in wastewater (ng/L to a few µg/L), TrOC biodegradation is most likely to occur via co-metabolism (Tran et al., 2013). In other words, TrOCs are usually not utilised by microorganisms as primary substrate for growth. Instead, TrOCs are biodegraded when other carbon sources are available (*e.g.*, biodegradable COD in wastewater). Co-metabolic pathways may lead to the formation of metabolites that participate in metabolic reactions that result in the complete mineralisation of TrOCs (Tran et al., 2013). Nevertheless, biodegradation of pure TrOCs by bacterial metabolism, *i.e.* the utilisation of TrOCs as the primary substrates for bacterial growth, has also been shown in pure bacterial cultures and batch tests using activated sludge (Quintana et al., 2005).

The biodegradability of TrOCs depends on the chemical structure of the compounds. Compounds that have highly branched or short hydrocarbon chains and halogen, sulphonate, methoxy, and nitro moieties are generally recalcitrant (Birkett and Lester, 2003; Hai et al., 2011b). Moreover, the biodegradation of TrOCs is impacted by their sorption potential. The sorption of biodegradable compounds on bacterial surfaces facilitates their reaction with extracellular enzymes and uptake into cells (Birkett and Lester, 2003), but the sorption of non- or slowly-biodegradable compounds on sludge decreases their bioavailability and causes contamination build-up (Barret et al., 2012; Wijekoon et al., 2013). Barret *et al.* (2012) developed a model for TrOC co-metabolism in anaerobic sludge, and showed that compounds partitioned in the aqueous phase undergo biodegradation. Wijekoon *et al.* (2013) reported that hydrophobic and persistent TrOCs significantly accumulate in the MBR.

TrOC biodegradation sometimes result in the formation of toxic metabolites that have more adverse impact on the environment and human health than their parent compounds (Birkett and Lester, 2003; Tran et al., 2013). For instance, the aerobic biodegradation of long chain nonylphenol ethoxylates into short chain nonylphenol ethoxylates followed by the anaerobic degradation of the ethoxylate groups increases the concentration of highly toxic nonylphenol in sludge (Birkett and Lester, 2003; Patureau et al., 2008). Metabolites from the biodegradation of pharmaceuticals (Lahti and Oikari, 2011; Quintana et al., 2005), linear alkyl benzene sulfonates (García et al., 2005), and UV filters (Ramos et al., 2015) in sludge were also identified, as well as products from bioconversion among hormones (Chawla et al.,

2014; Samaras et al., 2014). However, limited information is available on the biodegradation pathways of TrOCs due to the wide variety of compounds in real wastewater, which makes it difficult to relate detected metabolites to the parent compounds (Nguyen et al., 2015; Ramos et al., 2015). The complexity of the sludge matrix also creates issues in sample extraction and analysis (Barnabé et al., 2009; Ramos et al., 2015). Therefore, it is beneficial to observe biodegradation of individual compounds by pure cultures to understand potential reaction pathways in sludge. For instance, some biodegradation products of di(2-ethylhexyl)phthalate and bisphenol A exhibit toxicity and/or estrogenicity (Barnabé et al., 2009). It is also useful to perform toxicity or estrogenic activity assays to evaluate the efficiency of treatment procedures and potential hazards of effluent and sludge to be disposed or re-used (Muller et al., 2008; Nguyen et al., 2015).

2.7.1.3 Abiotic TrOC transformation

Abiotic transformation has minimal impact on the removal of TrOC from wastewater and sludge (Barnabé et al., 2009). A small fraction can be removed via abiotic mechanisms such as volatilisation, hydrolysis, thermal degradation, and photolysis. Volatilisation may occur at ambient temperatures for hydrophilic compounds with relatively low solubility in water and high Henry's law constant (k_H) at liquid-gas interfaces, e.g. surface of aeration tanks (Birkett and Lester, 2003; Hamid and Eskicioglu, 2012; Suárez et al., 2012), or at elevated temperatures, e.g. during composting or thermophilic digestion (Gibson et al., 2007; Muñoz et al., 2014; Patureau et al., 2008). Most TrOCs have low k_H and thus TrOC removal by volatilisation in WWTPs has only been observed for a few compounds (e.g. fragrances and polycyclic hydrocarbons) at minimal quantities (Patureau et al., 2008; Suárez et al., 2012). Hydrolysis has potential to occur in aqueous environments (Birkett and Lester, 2003), but thus far negligible TrOCs hydrolysis in sludge matrices has been observed (Batt et al., 2007; Styrishave et al., 2011). Thermal degradation of TrOCs has not been substantiated in literature, although the disappearance of pharmaceuticals during sludge drying (*e.g.*, 180° C) has been attributed to this mechanism (Lillenberg et al., 2010). Direct or indirect photolysis degrades some TrOCs (*e.g.*, pesticides) in aqueous solutions (Reddy and Kim, 2015), and UV disinfection performed after secondary or tertiary treatment has been observed to remove up to 20% of pharmaceuticals in the effluent (Salgado et al., 2012). However, photolysis generally has minimal impact on TrOC removal from sludge matrices (Barnabé et al., 2009; Batt et al., 2007).

2.7.2 Effects of various factors on fate of TrOCs in biological wastewater treatment

Multiple factors including TrOC properties and reactor operation conditions can simultaneously impact the sorption and biodegradation of TrOCs in sludge (Figure 2.7). These factors must be considered when elucidating the fate of TrOC in wastewater treatment and designing control strategies for TrOC abatement in effluent or biosolids.

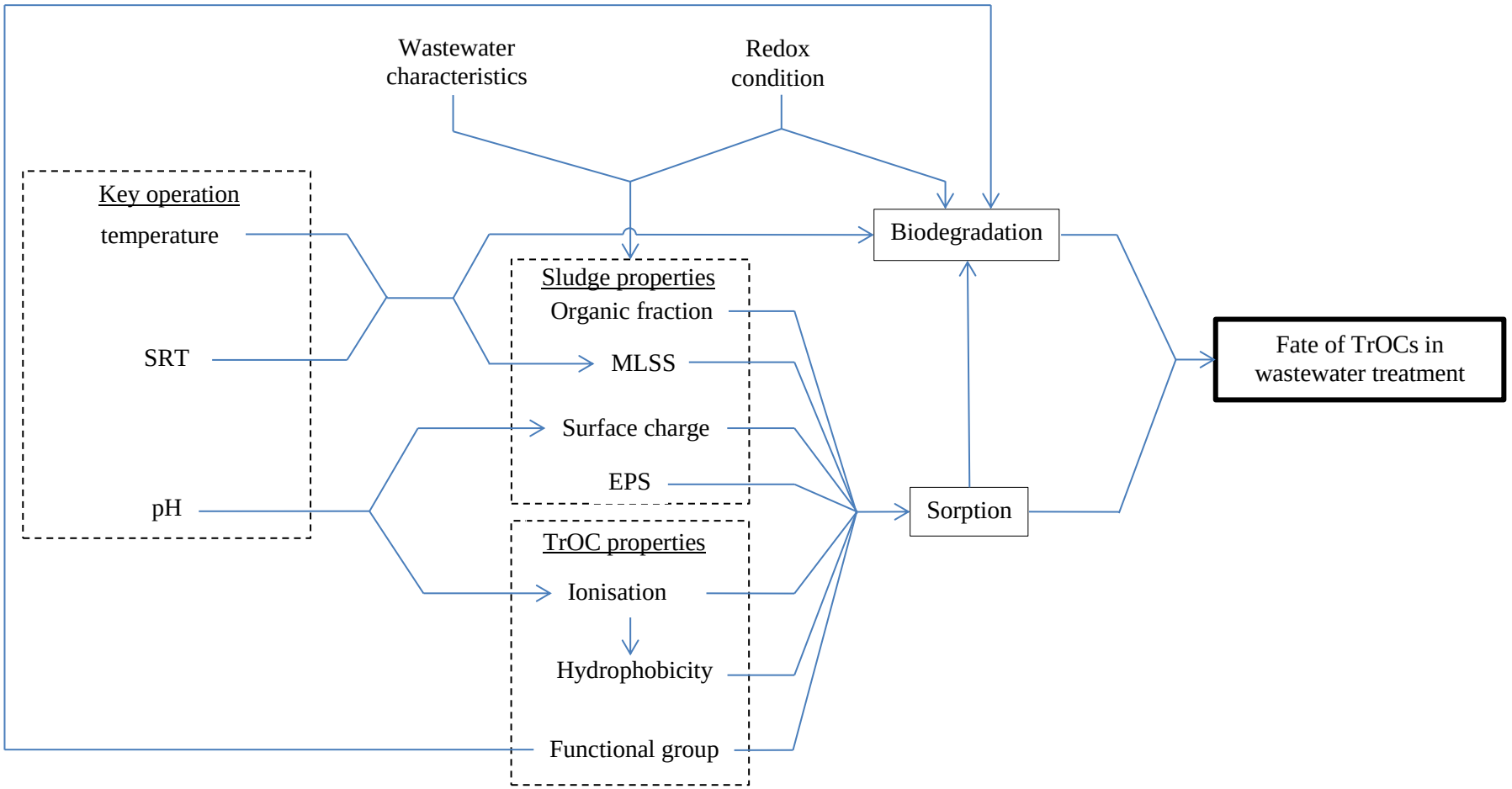

Figure 2.7. Schematic diagram depicting the key operation conditions impacting the accumulation of TrOCs on activated sludge

2.7.2.1 Effect of redox condition

Biodegradation occurs under different redox conditions (aerobic, anoxic, and anaerobic), with each condition offering biodegradation pathways that may not be available in others due to its distinct microbial consortia. Aerobic treatment generally results to high biodegradation of TrOCs through the action of heterotrophic bacteria and autotrophic ammonia-oxidizing organisms (AOOs) (Tran et al., 2013). Using 17α-ethinylestradiol as a model pollutant, independent researchers showed that heterotrophs and AOOs break down TrOCs using the enzymes catechol dioxygenase (Khunjar et al., 2011) and ammonia monoxygenase (Yi et al., 2006), respectively. AOOs were identified as the key microorganisms responsible for the biodegradation of certain pharmaceuticals (*e.g.*, roxithromycin, erythromycin, and iopromide) (Batt et al., 2006; Dorival-García et al., 2013; Suarez et al., 2010), although cooperative biodegradation of 17α-ethinylestradiol, trimethoprim, and their metabolites by both heterotrophs and AOO was also observed (Khunjar et al., 2011). Interestingly, heterotrophic bacteria belonging to the same group can have different TrOC biodegradation capacity. Pure cultures of *Rhodococcus rhodochrous* completely degraded 17α-ethinylestradiol, whereas pure cultures of *R. equi, R. erythropolis,* and *R. zopfii* were only able to degrade about 60% of the same compound (Larcher and Yargeau, 2013).

Many TrOCs undergo greater biodegradation under aerobic than anoxic conditions (Dorival-García et al., 2013; Phan et al., 2014; Stadler et al., 2015; Suarez et al., 2010). Suarez *et al.* (2010) and Dorival-García *et al.* (2013) demonstrated that compounds such as dicloflenac, naproxen and roxithromycin were recalcitrant under anoxic conditions, but had moderate to high removal (*e.g.*, 14.9-60%) under aerobic conditions. Phan *et al.* (2014) observed that most of the 30 diverse TrOCs tested were biodegraded under aerobic conditions, and only a few TrOCs were degraded under anoxic or anaerobic conditions. Nonetheless, there are also reports of TrOCs having similar or higher biodegradation in anoxic reactors in comparison with aerobic reactors under specific circumstances such as high SRT or low DO concentration. Suarez *et al.* (2010) noted that synthetic musks (*e.g.*, tonalide and galaxolide) achieved high biodegradation under anoxic conditions with SRT>20 d. Hai *et al.* (2011a) and Stadler *et al.* (2015) observed comparable or higher biodegradation of sulfamethaxozale under near-anoxic conditions (*e.g.,* DO concentration < 0.5 mg/L), probably because both aerobic and anoxic co-metabolic pathways were available under those conditions. Therefore, a systematic combination of aerobic and anoxic treatment can enhance TrOC removal. Phan *et al.* (2014) found that anoxic condition promoted the sorption of hydrophobic TrOCs on

sludge, which possibly facilitated their biodegradation when sludge was recirculated between aerobic and anoxic conditions. Furthermore, Phan *et al.* (2015) observed that a full-scale MBR with multiple aerobic and anoxic zones showed higher and more stable removal of TrOCs than a pilot-scale MBR containing only aerobic and anoxic reactors. This emphasises the effect of varying DO levels in enhancing the sorption-biodegradation mechanism for TrOC removal.

Anaerobic treatment is marked by unique biotransformation pathways, such as reductive dehalogenation of chlorinated compounds, bioconversion of natural hormones, and enantioselective biodegradation (Birkett and Lester, 2003; Gasser et al., 2012; Joss et al., 2004). Reductive dehalogenation of six chlorophenols was observed in upflow anaerobic sludge blanket reactors. The reaction involved either hydrogenolysis (*i.e.*, the substitution of chlorine atoms with hydrogen) or vicinial reduction (*i.e.*, the removal of two halogens from adjacent carbon atoms resulting to the formation of a double bond) (Birkett and Lester, 2003). Bioconversion of hormones was observed in anaerobic wastewater and sludge treatment (Joss et al., 2004; Zhang et al., 2013). Joss *et al.* (2004) reported that estrone was converted to 17β-estradiol exclusively under anaerobic conditions. Paterakis *et al.* (2012) reported that the bioconversion among hormones led to the formation of 17β-estradiol during anaerobic digestion. This suggests that although anaerobic treatment results in moderate to high estrogen removal (Joss et al., 2004; Zhang et al., 2013), it may result in the bioconversion of hormones and metabolites that can increase estrogenicity of sludge. Finally, there is evidence showing the enantioselective biodegradation of chiral TrOCs (Gasser et al., 2012). Gasser *et al.* (2012) observed that batch anaerobic treatment of the drug *R,S*-venlafaxine and its metabolite *R,S*-O-desmethylvenalfaxine produced degradation products with different enantiomeric distribution than that of aerobic treatment. Wang *et al.* (2014) noted high removal (*e.g.*, >93%) of five polycyclic musks in a laboratory-scale anaerobic MBR through biodegradation, but did not see enantioselectivity in the reactions.

2.7.2.2 Effect of pH

The interactions of TrOC and sludge at neutral pH have been extensively studied (Clara et al., 2005; Suarez et al., 2010). Nonetheless, secondary treatment may occur at higher or lower pH due to the characteristics of wastewater or addition of chemicals for sludge conditioning (Calace et al., 2002; Clara et al., 2004). In such cases, the sorption and biodegradation of ionisable compounds is expected to change depending on their acid dissociation constant

(pK_a) and the surface charge of sludge. Urase and Kikuta (2005) found that reducing reactor pH from 7 to 5 increased the sorption of TrOCs containing carboxylic acid groups (*e.g.*, fenoprop) because the un-dissociated and neutral forms of the compounds predominated at lower pH. Meanwhile, Hörsing *et al.* (2011) demonstrated that increasing mixed liquor pH from 6 to 8 caused 10-20% change (decrease or increase) in the sorption of pharmaceuticals containing nitrogen or amine groups. Clara *et al.* (2004) observed in batch experiments that bisphenol A (pKa=10.2) was desorbed from sludge when pH was increased from 7 to 9-12. Notably, pH is not expected to influence the sorption behaviour of non-ionisable TrOCs. Tadkaew *et al.* (2010) varied the mixed liquor pH of an MBR from 5 to 9 and found that the removal of ionisable TrOCs changed with pH, whereas those of non-ionisable TrOCs were independent of pH.

2.7.2.3 Effect of SRT

SRT affects sludge concentration and properties such as EPS composition and hydrophobicity, which may have opposing influence on TrOC sorption (Hai et al., 2014; Liao et al., 2001). Hence, contradictory results have been reported in literature. For instance, Kim *et al.* (2005) observed that decreasing SRT from 10 to 3 d decreased the MLSS of sludge and consequently reduced the sorption of tetracycline by 9%. This probably occurred because there were fewer sorption sites at lower sludge concentration. On the other hand, Banihashemi and Droste (2014) observed that decreasing SRT from 15 to 5 d increased MLSS concentration due to faster microbial growth rate, and there was no correlation between MLSS concentration and sorption of hormones and pharmaceuticals. These findings imply that MLSS concentration is not the only SRT-dependent factor affecting sludge-TrOC interactions (Liao et al., 2001). Lee *et al.* (2003) suggested that increasing SRT may increase EPS concentration, and consequently increase sludge hydrophobicity and affinity towards organic pollutants. The removal efficiencies of TrOC with high sorption (*e.g.*, bisphenol A, estrone, and 17β-estradiol; log D > 3 at pH 8) and moderate sorption (*e.g.*, estriol and bezafibrate; 2 < log D < 3 at pH 8) have been found to increase with SRT in different laboratory- (SRT=2 to 68 d) and full-scale (SRT=0.6 to 550 d) CAS and MBR plants (Clara et al., 2005). Nonetheless, various studies also found that the sorption of some hydrophobic compounds (e.g., 17β-estradiol; log D = 4.52 at pH 7) were unaffected by SRT (Clara et al., 2005; Hyland et al., 2012; Stasinakis et al., 2010). Similarly, Hyland *et al.* (2012) did not observe a correlation between SRT and sorption of various ionisable TrOCs. Stasinakis *et al.* (2010) demonstrated that varying SRT (e.g. 3-20 d) had no impact on the sorption of triclosan

and bisphenol A, although nonylphenol exhibited high sorption at 3 d. Further investigation must be performed to elucidate the impact of SRT on other sludge properties, such as floc size and density, and their implications on TrOC sorption.

TrOC biodegradation may increase with SRT due to the (1) increase in sludge biodiversity, and (2) diversification in the metabolic activity of microorganisms due to unavailability of preferred substrate (Hai et al., 2014). Clara *et al.* (2005) reported that SRT>10 d was sufficient to degrade most TrOCs and achieve low effluent TrOC concentrations, although recalcitrant compounds such as carbamazepine were unaffected by operation conditions. Tambosi *et al*. (2010) observed that the biodegradation of TrOCs increased when the SRT of an MBR was increased from 20 to 30 d. However, other researchers found that SRT variation at a low (*e.g.*, 3-20 d) (Stasinakis et al., 2010) and high (*e.g.*, 10-80 d) (Joss et al., 2004) range did not have any impact on the biodegradation of TrOCs.

2.7.2.4 Effect of temperature

As an enthalpy-driven process, the sorption of TrOCs on sludge due to hydrophobic and electrostatic interactions is temperature-dependent (ten Hulscher and Cornelissen, 1996). Temperature also affects biodegradation kinetics and microbial communities (LaPara et al., 2001). Laboratory-scale studies demonstrated that temperature variation, which occurs in full-scale plants due to seasonal changes (Tchobanoglus et al., 2003), may affect TrOC sorption and biodegradation. For instance, Zeng *et al.* (2009) reported that the sorption of 17α-ethinylestradiol on inactivated aerobic and anaerobic sludge was greater at 10 °C than 30 °C because Gibbs free energy (ΔG°, an indicator of the spontaneity of the process) decreased as temperature decreased. Hai *et al.* (2011) observed that the removal of hydrophobic TrOCs (log D>3) was stable at 10-30 °C, but was unstable and lower at 45 °C. Moreover, the removal of hydrophilic TrOCs (log D<3) varied considerably at 10-30 °C probably because of unstable biodegradation.

Thermophilic secondary treatment of high-strength wastewaters such as those from the pharmaceutical industry may show enhanced organic biodegradation along with low sludge yield (LaPara et al., 2001). However, it may cause a decline in the removal of hydrophobilic TrOCs as demonstrated by the study of Hai *et al.* (2011). Wijekoon *et al.* (2014) reported higher TrOC removal in a thermophilic MBR combined with membrane distillation relative to an MBR alone, but the improvement was attributed to TrOC rejection by membrane distillation rather than enhanced organic biodegradation in the MBR under thermophilic

conditions. Thus far, the conceptual advantage of thermophilic over mesophilic treatment in terms of TrOC removal has not been demonstrated in literature.

2.7.2.5 Effect of sludge concentration

TrOC sorption was to increase with MLSS (Auriol et al., 2006; Kim et al., 2005) probably because higher MLSS provides a greater number of sorption sites for hydrophobic interactions. Li *et al.* (2005) found that 17β-estradiol biodegradation increased with MLSS (0.4 to 1.7 g/L) in batch experiments, and likewise Shariati *et al.* (2010) noted acetaminophen biodegradation was higher at greater MLSS in an MBR (2-15 g/L) (Li et al., 2005; Shariati et al., 2010). On the other hand, Li *et al.* (2011) did not observe any impact of MLSS (1-15 g/L) on carbamazepine removal of an MBR. Identifying an optimal MLSS value or range for TrOC biodegradation is difficult since only a few studies have focused on the subject, and the few available studies assessed the removal of different types of TrOCs.

2.7.3 Fate of TrOCs in sludge handling and treatment units

2.7.3.1 Aerobic digestion

Aerobic digestion involves the treatment of thickened sludge in a completely mixed aerated reactor that is commonly used by small WWTPs (< 22 ML/day) (Tchobanoglus et al., 2003) and is notable for greater biodegradation of TrOCs such as nonylphenol/nonylphenol ethoxylates, hormones, and polycyclic hydrocarbons compared to anaerobic digestion (Esperanza et al., 2007; Holbrook et al., 2002; Ömeroğlu and Sanin, 2014; Trably and Patureau, 2006). Reports to date usually reveal that TrOCs such as nonylphenol have minimal impact on the organic matter and volatile solids removal efficiency of aerobic digestion (Ömeroğlu and Sanin, 2014), but the effect of other compounds is yet to be investigated. Studies suggest that TrOC biodegradation in aerobic digestion is strongly dependent on temperature and SRT (Marti and Batista, 2014; Trably and Patureau, 2006). Trably and Patureau (2006) reported that the biodegradation of polycyclic aromatic hydrocarbons increased from 50 to 80% when the aerobic digester temperature increased from 35 to 55^{o}C. However, they observed abiotic losses at higher temperatures due to volatilisation. They also noted an increase in polycyclic aromatic hydrocarbon biodegradation by the addition of methanol to sludge, which enhanced the dissolution of TrOCs in the liquid phase. Marti and Batista (2014) emphasised that there was high removal of estrogens when the SRT (*e.g.*. 40-60 d) of aerobic digesters is relatively long due to an extended reaction time.

2.7.3.2 Anaerobic digestion

The industrial demand for anaerobic digestion has increased considerably due to its relatively low operation cost and potential to generate energy (Gao et al., 2014; Nghiem et al., 2014a; Nghiem et al., 2014b). However, the persistence of TrOCs in sludge may pose problems to this process. First, methanogens are susceptible to organic pollutants such as chlorophenols, halogenated aliphatics, and N-substituted aromatics (Chen et al., 2008). Second, full-scale anaerobic digesters generally have negligible or poor biodegradation of TrOCs (Golet et al., 2003; Holbrook et al., 2002; Marti and Batista, 2014; Narumiya et al., 2013; Sanz et al., 2003), and reports of high TrOC removal are limited to laboratory-scale anaerobic digesters (Carballa et al., 2007b; Esperanza et al., 2007). Third, some anaerobic co-metabolic pathways may produce more potent pollutants. For instance, the formation of nonylphenol from nonylphenol ethoxylates and that of 17β-estradiol from estrone have been observed (Chawla et al., 2014; Patureau et al., 2008; Samaras et al., 2014). This may have serious implications on the toxicity and/or estrogenicity of biosolids. The formation of estrogenic byproducts is consistently observed even in the anaerobic digestion of other materials such as animal manure (Combalbert et al., 2012; Massé et al., 2014; Zhang et al., 2014).

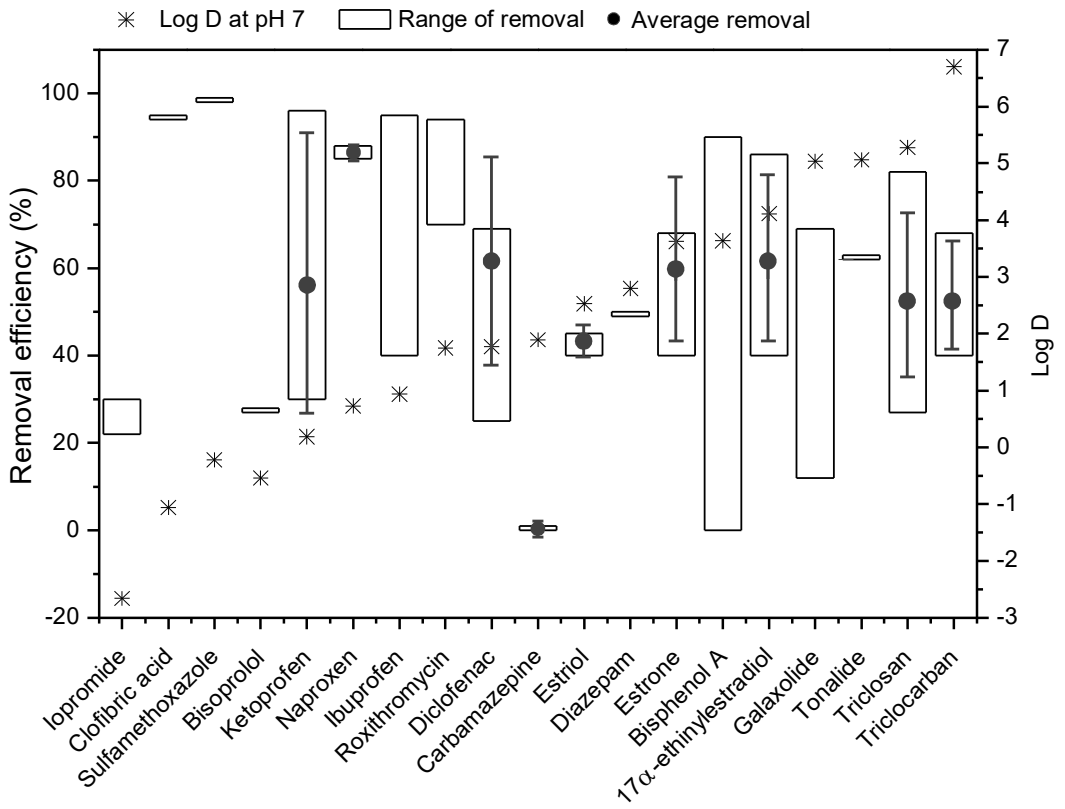

Figure 2.8 TrOC removal by anaerobic digestion superimposed with log D at pH 7. Error bars represent variation in removal efficiencies reported by different independent studies (n = number of samples): 17α-ethinylestradiol (3), bisoprolol (1), bisphenol A (2), carbamazepine (4), clofibric acid (1), diazepam (2), diclofenac (4), estriol (4), estrone (4), galaxolide (2), ibuprofen (2), iopromide (2), ketoprofen (3), naproxen (3), roxithromycin (2), sulfamethoxazole (2), triclocarban (2), triclosan (4). Data source: (Carballa et al., 2007a; Carballa et al., 2007b; de Graaff et al., 2011; Esperanza et al., 2007; Lahti and Oikari, 2011; Limam et al., 2013; Muller et al., 2010; Narumiya et al., 2013; Ogunyoku and Young, 2014; Paterakis et al., 2012; Reyes-Contreras et al., 2011; Samaras et al., 2014; Zhou et al., 2013)

It was highlighted in Section 2.7.1.1 that the TrOC removal efficiency of wastewater treatment can be predicted using the hydrophobicity of TrOCs as represented by log D. However, during anaerobic digestion, a relationship between TrOC removal and log D could not be derived from available literature (Carballa et al., 2007a; Carballa et al., 2007b; de Graaff et al., 2011; Esperanza et al., 2007; Lahti and Oikari, 2011; Limam et al., 2013; Muller et al., 2010; Narumiya et al., 2013; Ogunyoku and Young, 2014; Paterakis et al., 2012; Reyes-Contreras et al., 2011; Samaras et al., 2014; Zhou et al., 2013). TrOCs with high log D (*e.g.*, 17α-ethinylestradiol and triclosan) may exhibit lower removal than TrOCs with lower log D (*e.g.*, ketoprofen and dicloflenac), and *vice versa* (Figure 2.8). Narumiya *et al.* (2013) demonstrated that although TrOC sorption on anaerobic digester sludge still depends on its hydrophobicity and/or charge at a given pH, it does not seem to have correlation with

biodegradation. Variation in TrOC removal in literature (Figure 2.8) may be due to varying operation conditions and solids destruction efficiency. Studies suggest that TrOC removal in anaerobic digestion could be correlated with solids destruction, which potentially increases the bioavailability of the compounds. For example, Patureau *et al.* (2008) and Trably *et al.* (2003) observed that the removal of nonylphenol ethoxylates and polycyclic aromatic hydrocarbons increased with the TS removal of the anaerobic digester, possibly due to the desorption of the compounds from destroyed flocs and loss of sorption sites. However, Marti and Batista (2014) speculated that although estrogen desorbs as sludge flocs are destroyed, it re-sorbson the remaining flocs leading to the accumulation of estrogen in sludge.

Anaerobic digester operation conditions (*e.g.,* temperature, type of sludge, SRT) could affect sorption, reaction rates, and microbial community, and thus have potential to impact on the fate of TrOC. The effect of temperature differs with the type of TrOC (Figure 2.9). Studies concur that thermophilic digestion favours the removal of polycyclic aromatic hydrocarbons (Barret et al., 2012; Benabdallah El-Hadj et al., 2007; Trably et al., 2003) especially those with higher molecular weight (Trably et al., 2003), but different trends are reported for hormones, nonylphenol/nonylphenol ethoxylates, and pharmaceuticals (Carballa et al., 2007a; Esperanza et al., 2007; Lahti and Oikari, 2011; Muller et al., 2010; Paterakis et al., 2012; Samaras et al., 2014). For instance, Paterakis *et al.* (2012) reported that increasing digester temperature from mesophilic (35±0.2 °C) to thermophilic (55±0.2 °C) enhanced the removal of estrone, but increased the bioconversion among hormones leading to a decrease in the removal of estriol and significant formation of 17β-estradiol. The same study also observed that thermophilic conditions enhanced the biodegradation rate of small nonylphenol ethoxylates (e.g. NP_1E or NP_2E) by nearly 100%, but only increased the removal of large nonyl polyethoxylates (e.g. NP_nE where n=3-12) by 23%. On the other hand, Patureau *et al.* (2008) observed only a 20% increase in nonylphenol and nonylphenol monoethoxylate removal by increasing digestion temperature from mesophilic to thermophilic, probably because of different experimental conditions (*e.g.,* SRT). Since mesophilic and thermophilic digestion each provides unique biodegradation pathways, a temperature-phased anaerobic digestion configuration has potential to improve overall TrOC removal. Samaras *et al.* (2014) found that two-stage thermophilic-mesophilic digestion had higher removal of triclosan, bisphenol A and nonylphenol than either single-stage mesophilic or thermophilic digestion, but did not improve the removal of other compounds such as ibuprofen and naproxen. Notably, Carballa *et al.* (2007a) also did not observe changes in the removal of ibuprofen,

naproxen, and other pharmaceuticals due to thermophilic digestion, indicating that the biodegradation of such compounds are not dependent on temperature.

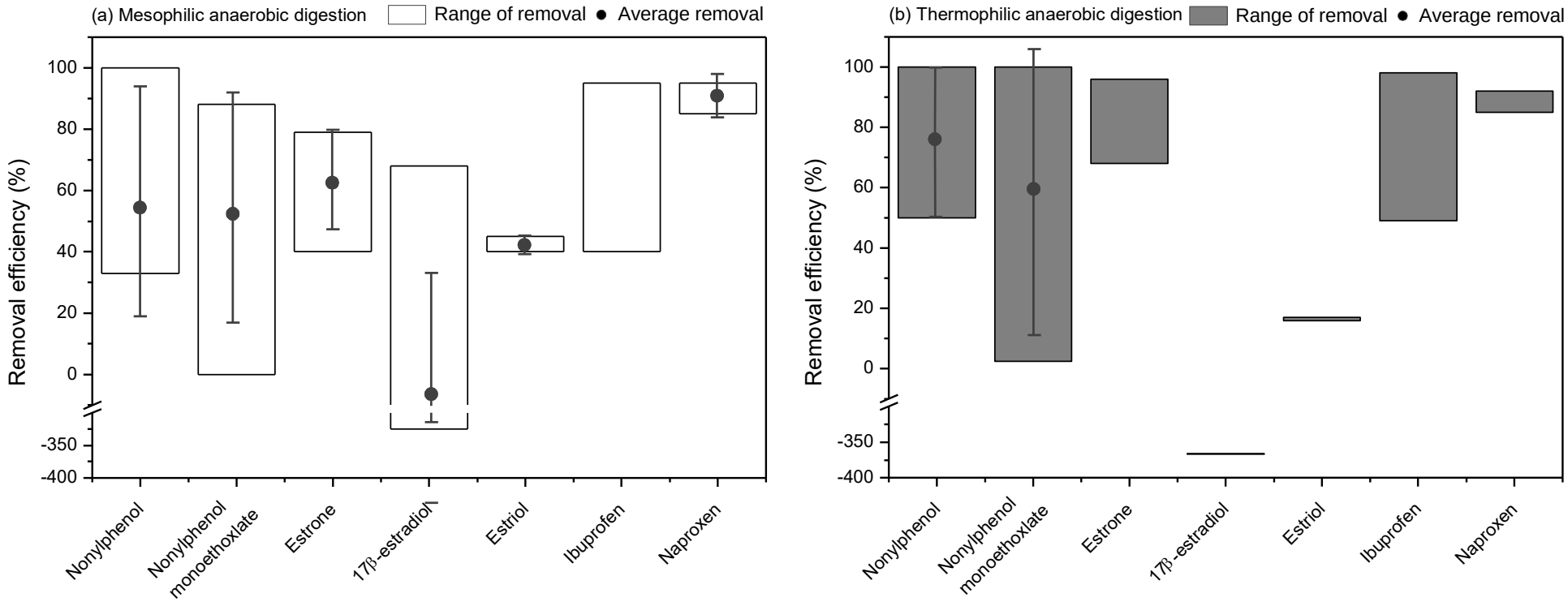

Figure 2.9. TrOC removal by mesophilic and thermophilic anaerobic digestion. Error bars represent variation in removal efficiencies reported by different independent studies ((n, m) = number of samples in *m* mesophilic and, *n* thermophilic condition, respectively): 17β-estradiol (4, 2), estriol (4, 2), estrone (4, 2), ibuprofen (2, 2), naproxen (3, 2), nonylphenol (3, 3), nonylphenol monoethoxylate (3, 3). Data source: (Carballa et al., 2007a; Esperanza et al., 2007; Lahti and Oikari, 2011; Muller et al., 2010; Paterakis et al., 2012; Samaras et al., 2014)

Primary (collected from the primary settling tank) and secondary sludges (WAS collected from the secondary settling tank) have different composition and floc properties (Tchobanoglus et al., 2003). Paterakis *et al.* (2012) reported that the removal of estrogen and nonylphenol ethoxylates from mixed sludge was 20-80% higher than that of primary sludge, but a clear explanation for this was not provided. SRT has been found to have significant impact on the sorption and biodegradation of TrOCs during wastewater treatment (Section 2.3.2), but so far only a few studies have investigated the effect of SRT on TrOC removal of anaerobic digestion. Carballa *et al.* (2006) covered a range of SRTs under mesophilic (SRT=10, 20, and 30 d) and thermophilic (SRT=6, 10, and 20 d) conditions, but did not see significant difference in the removal of various hormones and pharmaceuticals due to SRT. On the other hand, Hamid and Eskicioglu (2013) found that amount of estrone and androstenedione in the supernatant of a thermophilic anaerobic digester increased by 1.2-1.5 and 2-4 times, respectively, when SRT was increased from 5 to 20 d probably due to bioconversion among hormones or other compounds (*e.g.*, sterols).

2.7.3.3 Alkaline stabilisation

Alkaline treatment is a relatively inexpensive process that involves the addition of materials such as lime, fly ash, or cement kiln dust to raise sludge pH to 12 for one day or longer. The drastic change in pH is expected to alter the sorption behaviour of ionisable TrOC. Ivashechkin *et al*. (2004) found that increasing sludge pH to 12.4 using calcium hydroxide caused desorption of BPA (pKa=10.3) from flocs. Likewise, Kim *et al.* (2013) observed that the concentration of polybrominated diphenyl ethers in sludge decreased after alkaline treatment because of dilution. Conceptually, the partitioning of TrOCs in the aqueous phase may enhance their removal from the sludge matrix after dewatering or increase their bioavailability in further sludge treatment (*e.g.*, aerobic or anaerobic digestion). Nonetheless, Kouloumbos *et al*. (2008) reported that the removal of radiolabelled nonylphenol (pKa=10.7) from the solid phase of sludge was minimal (*e.g.*, 1.3%) after alkaline stabilisation (*e.g.*, treatment at pH 11 using calcium hydroxide) and centrifugation. The impact of alkaline treatment became apparent when sludge was applied on soil wherein the leaching of nonylphenol increased. Further study is necessary to confirm this trend as the extractability other non-biodegradable organic components of sludge (*e.g.*, humic acids and lipids) increased after alkaline stabilisation (Li et al., 2009). Furthermore, Carballa *et al.* (2006) found that alkaline pre-treatment of mixed sludge (70:30 by volume of primary and

secondary sludge) at pH 12 for 24 hours did not enhance the pharmaceutical removal efficiency of a laboratory-scale anaerobic digester. On the other hand, alkaline post-treatment of sludge may enhance the transmission of TrOCs from biosolids to receiving soils and have implications on the TrOC biodegradation pathways in the soil matrix (Citulski and Farahbakhsh, 2010).

2.7.3.4 Conditioning and dewatering

During dewatering by physical or thermal treatment, moisture is removed from sludge such that a 'cake' with 20% TS or more is produced to increase the performance of additional sludge stabilisation procedures (*e.g.*, aerobic digestion) and minimise the cost of sludge handling and transport (Tchobanoglus et al., 2003). However, water molecules are tightly bound in sludge flocs due to their biological gel-like structure. Thus, sludge dewatering is commonly preceded by a chemical or thermal sludge conditioning step. In chemical sludge conditioning, materials such as lime, iron salts, and polymer are added into sludge to coagulate small particles into larger aggregates that have greater capacity to release water. In thermal sludge conditioning, sludge is heated to 230 to 290°C to evaporate water that is entrapped in sludge flocs (Mowla et al., 2013; Tchobanoglus et al., 2003).

Thus far, there is inconclusive data on the impact of dewatering on the fate of TrOCs in sludge. Some studies reported that dewatering by centrifugation or filter press increased the concentration of TrOCs in dewatered sludge (Marti and Batista, 2014; Muller et al., 2010). Muller *et al.* (2010) suggested that the increase in 17β-estradiol and 17α-ethinylestradiol levels after dewatering was due to the intense treatment conditions of the filter press (200 °C, 2 MPa) that enhanced the extractability of compounds. However, in that study, dewatering had no effect on other hormones such as estrone and estriol. Marti *et al.* (2014) reported that dewatering non-digested thickened sludge by centrifugation increased its total hormone concentration by 41%, but dewatering anaerobically digested sludge by the same procedure did not affect hormone concentration. Other studies demonstrated that dewatering by either centrifugation or filter pressing do not affect estrogen concentration of the solid phase of sludge (Braga et al., 2005; Muller et al., 2008). The impact of thermal dewatering on other types of TrOC is yet to be investigated in detail, but Lindberg *et al.* (2005) observed the thermal degradation of fluoroquinolones when sludge pellets underwent thin layer drying at 180 °C followed by moving

belt drying at 105 °C. It can be speculated that high temperature may cause evaporation of relatively volatile compounds such as nonylphenol and polychlorinated biphenyls.

As the final or penultimate step in the sludge treatment line, it is interesting to discover the impact of sludge dewatering and conditioning on the mineralisation, degradation, and mobility of TrOCs in biosolids after application on soil. Kouloumbos *et al.* (2008) monitored the biodegradation products, mineralisation products, and mobility of ^{14}C-labelled nonylphenol in soil amended with dewatered and conditioned anaerobically digested biosolids, and found that centrifuged biosolids was less penetrable to O_2 and have low bioavailability of nonylphenol to microorganisms in the soil. Meanwhile, sludge conditioned by lime had higher leaching potential of nonylphenol due to desorption at high pH, and sludge conditioned by acrylamide-based cationic polymer potentially have greater toxicity due to the formation of nitrophenol from the reaction of nonylphenol with the biodegradation products of the polymer (Kouloumbos et al., 2008).

2.7.4 Factors and other considerations that may have significant impact on fate of TrOCs in OSA

The fate of TrOCs in OSA has never been investigated, but useful information can be gleaned from available literature on the fate of TrOCs in conventional wastewater and sludge treatment units. The fate of TrOCs in OSA has potential to be significantly affected by the redox regimes in the system (*e.g.* aerobic/anoxic). Treatment under aerobic condition demonstrates the greatest potential to remove TrOCs and other estrogenic metabolites. Aerobic digestion of thickened sludge achieves high removal of TrOCs such as nonylphenol ethoxylates, hormones, and polycyclic aromatic hydrocarbons depending on reactor temperature and SRT (Sections 2.7.3.1 and 2.7.2.1). On the contrary, treatment under anoxic or anaerobic condition appears to achieve lower TrOC removal (Sections 2.7.2.1 and 2.7.3.2). In fact, according to some reports, anaerobic treatment may exacerbate the estrogenicity of sludge by facilitating biotransformation pathways that produce more estrogenic metabolites (Sections 2.7.3.2).

Of the various reactor operation conditions that were reviewed, SRT has the highest potential to influence the fate of TrOC in OSA (Section 2.7.2.3). SRT determines sludge concentration and hydrophobicity, which have implications on TrOC sorption and biodegradation. Additionally,

SRT have significant impact on microbial biodiversity that affects the metabolic activity of microorganisms. Therefore, a careful consideration of the fate of TrOCs at different SRTs in OSA must be performed.

OSA is expected to result in sludge biodegradation, which involves cell lysis and the destruction of volatile solids. Sludge biodegradation resulted in the liberation of sorbed TrOCs and the increase TrOC bioavailability when volatile solids are destroyed during anaerobic digestion (Section 2.7.3.2). Similar mechanisms may take place in OSA especially under oxygen- and substrate-deficient conditions. It is worthwhile to investigate these mechanisms have implications on the overall TrOC removal efficiency and the occurrence of TrOCs in the final sludge residue of OSA.

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CHAPTER 3: METHODOLOGY

3.1 OVERVIEW OF THE EXPERIMENTAL FRAMEWORK

This study systematically investigated the impact of three factors (iron salts dosage, SIR, and SRT) on sludge reduction in OSA. The approach of focusing on the factors affecting OSA performance provides valuable information that will guide WWTP operation to reduce biosolids production, and will be instrumental in elucidating the underlying mechanisms responsible for sludge reduction. Two laboratory-scale systems were operated in parallel: OSA and control CAS. These systems were fed with domestic sewage (to be described in Section 3.3). Although real wastewater can undergo significant temporal variations, it is critical to cultivate sludge with realistic growth rates and properties. Sludge reduction was assessed by comparing the sludge yield of OSA and control (to be described in Section 3.3). Wastewater and sludge properties were monitored (to be described in Section 3.5) to gain insight on biological reactions taking place in the reactors. Additionally, the microbial community structure of sludge was determined through DNA extraction followed by Illumina sequencing (to be described in Section 3.5.3). Finally,the concentration of TrOCs in wastewater and sludge were measured to determine the fate of TrOC (to be described in Section 3.5.4).

3.2 REACTOR CONFIGURATION AND OPERATION

The "OSA system" consisted of a sequencing batch reactor, SBR_{OSA} (5 L), attached to external aerobic/anoxic (2 L) and anoxic reactors (2 L) (Figure 3.1a). This configuration is distinct from those usually reported in literature that involves a singular external anoxic or anaerobic reactor (Saby *et al.*, 2003; Chon *et al.*, 2011). Meanwhile, the "control system" consisted of $SBR_{control}$ (5 L) attached to a single-pass aerobic digester (2 L) (Figure 3.1b). All of the reactors (Figure 3.2 and Figure 3.3) were immersed in a water bath with temperature of 25 ºC.

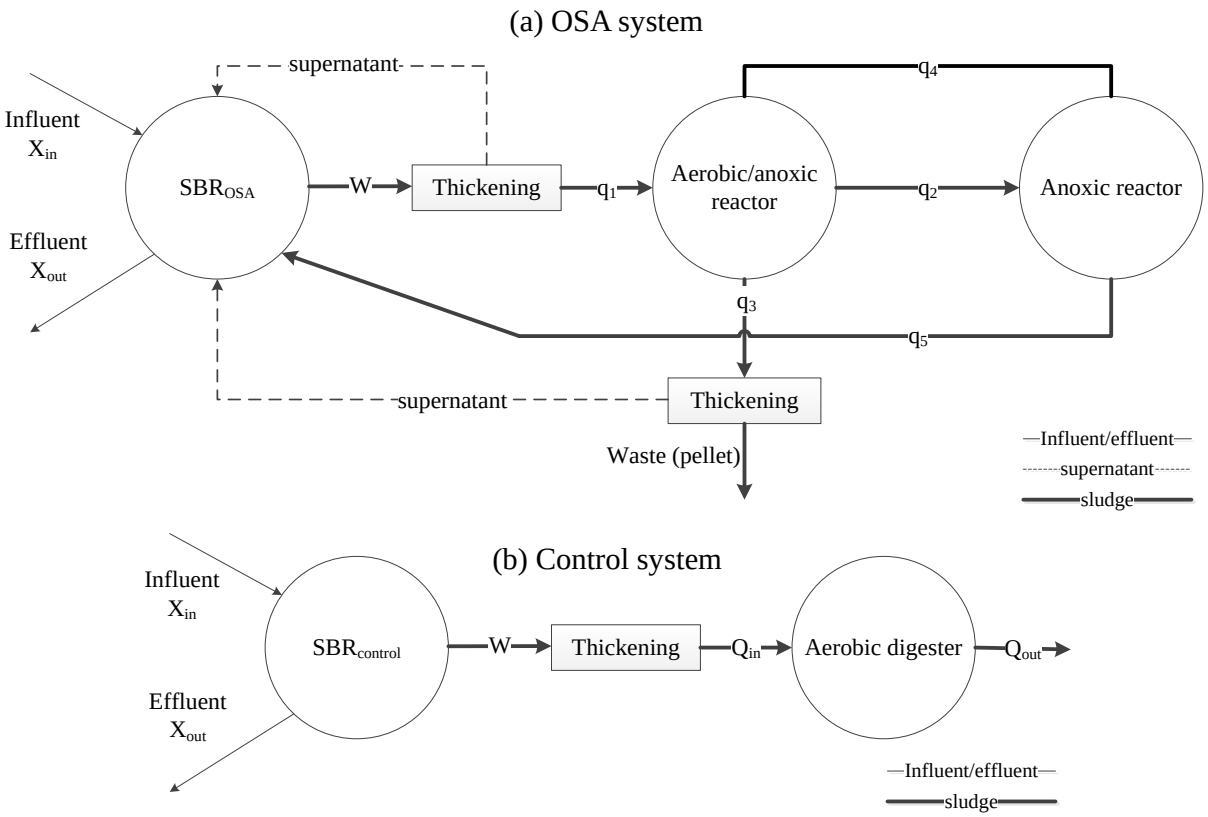

Figure 3.1. Schematic diagram of (a) the OSA system comprised of SBR_{OSA} attached to intermittently aerated (*i.e.*, aerobic/anoxic) and anoxic reactors, and (b) the control system comprised of $SBR_{control}$ attached to a single-pass aerobic digester

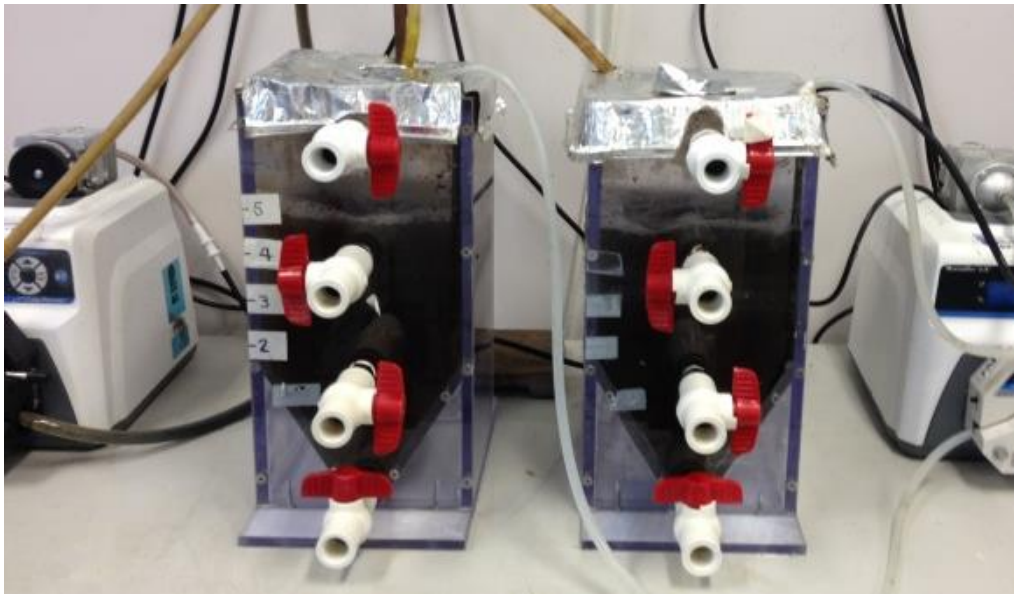

Figure 3.2. SBR_{OSA} (right) and $SBR_{control}$ (left). Note that the SBRs were taken out of the water bath (25 ºC) only for taking photos.

Figure 3.3. The external aerobic/anoxic (left) and anoxic (middle) reactors of the OSA system and the aerobic digester (right) of the control system.

3.2.1 OSA system

SBR_{OSA} (Figure 3.1a) was fed with domestic sewage. It was operated at 4 cycles/day and HRT of 12 hours. Each cycle comprised of 15 min of filling, 5 hours and 30 min of aeration, 1 hour of

settling, and 15 min of decanting. Wastewater was pumped in and out of SBR_{OSA} using peristaltic pumps (Masterflex, USA) controlled by electric timers. The SRT was maintained at 10 d by manual sludge wastage (*W*) (Figure 3.1a).

The aerobic/anoxic reactor (Figure 3.1a) was intermittently aerated (*i.e.*, 8/16 h aeration on/off) using an air diffuser placed at the bottom of the reactor. The anoxic reactor (Figure 3.1a) had no aeration and was kept airtight using a silicone-lined cap with inlet and outlet ports for feeding and sampling, respectively. Both of the reactors were continuously stirred using a magnetic stirrer.

The aerobic/anoxic reactor was manually fed with sludge from SBR_{OSA} thickened to 5-10 g/L (q_1) by centrifugation (Beckman Coulter, USA) at 3728x*g* (3750 revolutions per minute, RPM) and 25 ºC for 10 min. Thirty-three percent (33%) of sludge from the aerobic/anoxic reactor was transferred to the anoxic reactor (q_2) 67% was discharged to maintain a specific SRT (q_3). The sludge discharged from the aerobic/anoxic reactor was thickened to 16-24 g/L by centrifugation (Beckman Coulter, USA) at 3,267x*g* and 25 ºC for 10 min. The supernatant produced by the thickening step was returned to SBR_{OSA}, and the pellet was discarded. Sludge from the anoxic reactor was returned to the aerobic/anoxic reactor (q_4) and SBR_{OSA} (q_5). The flow rates of sludge in the external reactors were adjusted accordingly to maintain the desired SIR and SRT. Specific details are discussed in more detail in Chapters 4-8.

3.2.2 Control system

$SBR_{control}$ (Figure 3.1b) was fed with the same influent (domestic sewage) as SBR_{OSA}. Similar to SBR_{OSA}, $SBR_{control}$ was operated at 4 cycles/day and HRT of 12 hours. Each cycle comprised of 15 min of filling, 5 hours and 30 min of aeration, 1 hour of settling, and 15 min of decanting. Wastewater was pumped in and out of $SBR_{control}$ using peristaltic pumps (Masterflex, USA) controlled by electric timers. The SRT was maintained at 10 d by manual sludge wastage (W) (Figure 3.1b).

The aerobic digester (Figure 3.1b) of the control system was continuously aerated using an air diffuser placed at the bottom of the reactor and stirred using a magnetic stirrer. Its SRT was maintained by manual sludge wastage (Q_{out}). It was manually fed with sludge from $SBR_{control}$

thickened to 5-10 g/L by centrifugation (Beckman Coulter, USA) at 3728x*g* and 25 ºC for 10 min (Q_{in}). The supernatant produced by the thickening step was discarded.

3.2.3 Summary of the reactor operation

This study was divided into five main stages (Table 3.1). The SRT of SBR_{OSA} and $SBR_{control}$ (hereafter denoted as SRT_{SBR}) was maintained at 10 d throughout the experimental period to replicate conditions commonly applied in WWTPs receiving domestic sewage (Tchobanoglus et al., 2003). In the first stage, the effect of iron salt (ferrous chloride, $FeCl_2$) dosage on OSA performance was investigated by varying the concentration (none, 15, and 30 mg/L) added to the influent fed to both SBR_{OSA} and $SBR_{control}$. The highest sludge reduction (measured as the difference in sludge yield of SBR_{OSA} and $SBR_{control}$ under parallel conditions, to be described in more detail in Section 3.3) was observed when there was no ferrous chloride addition (to be discussed in Chapter 4). Therefore, ferrous chloride was not added to the influent in the succeeding stages of the study (Table 3.1). In the second stage, the SIR of OSA was varied (0, 11, 22, and 33%) to determine its impact on OSA performance. The highest sludge reduction was observed when SIR was 11% (to be discussed in Chapter 5), and therefore this condition was applied in the succeeding stages of the study (Table 3.1). The third, fourth, and fifth stages were performed simultaneously. The "external reactor" of the OSA system refers to aerobic/anoxic and anoxic reactors attached to SBR_{OSA} (Figure 3.1a), while that of the control system refers to the single-pass aerobic digester attached to $SBR_{control}$ (Figure 3.1b). The SRT of the external reactors (hereafter denoted as SRT_{ext}) was varied (10, 20, and 40 d) to determine its impact on sludge reduction. The highest sludge reduction was observed when SRT_{ext} was 20 d (to be discussed in Chapter 6). At each SRT_{ext}, sludge samples from all the reactors of OSA and control systems were obtained to analyse the microbial community structures (results to be discussed in Chapter 7). Also at each SRT_{ext}, samples from the influent, effluent, and sludge from all the reactors of OSA and control systems were obtained to determine the fate of TrOCs during wastewater treatment (to be discussed in Chapter 8).

Table 3.1. Summary of the operating parameters at different experimental stages of the study

Experimental stage	SRT_{SBR} [a] (d)	Ferrous chloride dosage (mg/L)	SIR (%)		SRT_{ext} (d) [c]	
			OSA	*Control*	*OSA (external aerobic/anoxic and anoxic reactor)*	*Control (aerobic digester)*
(1) Study on the effect of iron salts dosage	10	None, 15, and 30	16.5	Not applicable [b]	20	20
(2) Study on the effect of SIR	10	None	None, 11, 16.5, and 22	Not applicable [b]	20	20
(3) Study on the effect of SRT	10	None	11	Not applicable [b]	10, 20, and 40	10, 20, and 40
(4) Study on the microbial community structure	10	None	11	Not applicable [b]	20	20
(5) Study on the fate of TrOCs	10	None	11	Not applicable [b]	20	20

[a] SRT_{SBR} refers to the SRT of SBR_{OSA} and $SBR_{control}$; The value was maintained at 10 d throughout the experimental period.
[b] There was no sludge interchange in the control system
[c] SRT_{ext} refers to the SRT of the external reactors of the OSA (external aerobic/anoxic and anoxic reactor) and control (aerobic digester) systems.

3.3 DOMESTIC SEWAGE

Domestic sewage was obtained from Wollongong WWTP, a tertiary treatment plant operated by Sydney Water, New South Wales, Australia with discharge rate of 17.4 ML/day (SW, 2010). Unsettled or unsettled sewage was used for the experiments. The former was collected at the beginning of the primary sedimentation channel, whereas the latter was collected at the outlet of the same channel. They were collected weekly or fornightly and stored at 4 °C until use. Specific details on the sampling frequency and wastewater properties are discussed in Chapters 4-8.

3.4 MEASUREMENT OF SLUDGE REDUCTION

Sludge reduction was measured by comparing the sludge yield of SBR_{OSA} and $SBR_{control}$ under parallel operation conditions. The experimental sludge yield (*Y*) of a reactor was defined as

$$Y = \frac{P}{C} = \frac{g\ MLVSS}{g\ tCOD} \qquad \textbf{Equation 3.1}$$

where *P* is the sludge produced in terms of mixed liquor volatile suspended solids (MLVSS) and *C* is the substrate consumed in terms of COD. Sludge yield was derived from the slope of the linear regression of the cumulative sludge produced versus the cumulative substrate consumed. Cumulative values were obtained by incrementing the variations in sludge production and substrate consumption in previous sampling intervals (Chon *et al.*, 2011). The cumulative MLVSS produced by SBR_{OSA} (P_{SBROSA}) and $SBR_{control}$ ($P_{SBRcontrol}$) and a given time interval were quantified using a mass balance of biomass and shown in Equation 3.2 and Equation 3.3, respectively:

$$P_{SBR_{OSA}} = \Delta MLVSS_{SBR_{OSA}} \times V_{SBR_{OSA}} + \left(VSS_{out-SBR_{OSA}} \times X_{out} + MLVSS_{SBR_{OSA}} \times W - VSS_{in} \times X_{in} - MLVSS_{ANX} \times q_5\right) \times \Delta t \qquad \textbf{Equation 3.2}$$

$$P_{SBR_{control}} = \Delta MLVSS_{SBR_{control}} \times V_{SBR_{control}} + \left(VSS_{out-SBR_{control}} \times X_{out} + MLVSS_{SBR_{control}} \times W - VSS_{in} \times X_{in}\right) \times \Delta t \qquad \textbf{Equation 3.3}$$

where $MLVSS_{SBROSA,\ SBRcontrol,\ or\ ANX}$ are the biomass concentration (g/L) of SBR_{OSA}, $SBR_{control}$, or anoxic reactor, $V_{SBROSA\ or\ SBRcontrol}$ is the effective reactor volume (L), VSS_{in} is the volatile

suspended solids concentration (g/L) of the influent, $VSS_{out-\ SBRcontrol\ or\ SBROSA}$ is the volatile suspended solids concentration (g/L) of SBR_{OSA} or $SBR_{control}$ effluent, $X_{in\ or\ out}$ is flow rate (L/d) of the influent or effluent, W is the flow rate (L/d) of sludge wasted from the SBRs, q_5 is the flow rate (L/d) of sludge returned from the anoxic reactor to SBR_{OSA} (Section 2.2), and t is time (d) (Figure 3.1). Notably, VSS_{in} is deducted from the calculation of P_{SBROSA} and $P_{SBRcontrol}$ to discount the significant amount of volatile solids carried by real wastewater (*e.g.*, 0.1-0.5 g/L). $MLVSS_{ANX}$ is deducted from the calculation of P_{SBROSA} to discount the biomass that was recycled back to P_{SBROSA} from the external anoxic reactor (Figure 3.1a).

The amount of substrate consumed C was calculated according to the following equation:

$$C = \left(COD_{in} - COD_{out-SBR_{control}or\ SBR_{OSA}}\right) \times X_{in} \times \Delta t \qquad \textbf{Equation 3.4}$$

where COD_{in} and $COD_{out\text{-}SBRcontrol\ or\ SBROSA}$ are the COD concentration (g/L) of the influent and effluent of $SBR_{control}$ and SBR_{OSA}, respectively.

Additionally, the sludge yield of the control (combined $SBR_{control}$ and aerobic digester) and OSA (combined SBR_{OSA} and external aerobic/anoxic and anoxic reactors) systems were calculated. The synthesis of cells in the external reactors may occur even under limited substrate conditions when microorganisms consume products of cell lysis (Hao *et al.*, 2010), so those reactors alsos contribute to MLVSS production of the whole system. The MLVSS production of the OSA ($P_{OSA\ system}$) and control ($P_{control\ system}$) systems were calculated using Equation 3.5 and Equation 3.6, respectively.

$$P_{OSA\ system} = \Delta MLVSS_{SBR_{OSA}} \times V_{SBR_{OSA}} + \Delta MLVSS_{\frac{AE}{ANX}} \times V_{\frac{AE}{ANX}} + \Delta MLVSS_{ANX} \times V_{ANX} + \left(VSS_{out-SBR_{OSA}} \times X_{out} + MLVSS_{\frac{AE}{ANX}} \times q_3 - VSS_{in} \times X_{in}\right) \times \Delta t \qquad \textbf{Equation 3.5}$$

$$P_{control\ system} = \Delta MLVSS_{SBR_{control}} \times V_{SBR_{control}} + \Delta MLVSS_{AE} \times V_{AE} + \left(VSS_{out-SBR_{control}} \times X_{out} + MLVSS_{AE} \times Q_{out} - VSS_{in} \times X_{in}\right) \times \Delta t \qquad \textbf{Equation 3.6}$$

where $MLVSS_{AE/ANX\ or\ ANX}$ are the sludge concentration (g/L) of the aerobic/anoxic and anoxic reactors, $V_{AE,\ AE/ANX\ or\ ANX}$ is the effective volume (L) of the aerobic digester, aerobic/anoxic, or

anoxic reactor, q_3 is flow rate (L/d) of sludge wasted from the aerobic/anoxic reactor (Figure 3.1a), and Q_{out} is the flow rate (L/d) of sludge wasted from the aerobic digester (Figure 3.1b). Notably, the sludge interchanged within the external reactors and between SBR_{OSA} and external reactors were retained in the system hence it is not necessary to deduct those sludge flows from the calculation of P_{OSA}. The net substrate consumption of the system was calculated using Equation 3.4.

Sludge reduction was calculated as the difference in sludge yield of $SBR_{control}$ and SBR_{OSA}:

$$Sludge\ reduction\ (\%) = \frac{Y_{SBR_{control}} - Y_{SBR_{OSA}}}{Y_{SBR_{control}}} \times 100$$ **Equation 3.7**

3.5 ANALYTICAL TECHNIQUES

3.5.1 Analysis of wastewater and sludge

3.5.1.1 Solids concentration

The TSS and VSS of wastewater and the MLSS and MLVSS of sludge were measured according to Standard Method 2540 (Eaton *et al.*, 2005).

3.5.1.2 Sludge volume index

The sludge volume index (SVI) was measured using 1000 mL of sludge according to Standard Method 2710-D (Eaton *et al.*, 2005).

3.5.1.3 Total organic carbon and nitrogen

The Total organic carbon (TOC) and total nitrogen (TN) of wastewater was determined using a TOC/TN-VCSH analyzer (Shimadzu, Japan). The samples were centrifuged (Beckman Coulter, USA) at 3728x*g* and 25 ºC for 10 min to remove large solids, and the resulting supernatant was filtered using 1 µm filter paper prior to analysis.

3.5.1.4 Chemical oxygen demand

The total COD (tCOD) of wastewater was measured using Hach low range (LR) digestion vials that were heated in Hach DBR200 COD Reactor, and then analysed using Hach DR/2000 spectrophotometer (program number 430 COD LR measuring absorbance at 420 nm) according

to US-EPA Standard Method 5220. The soluble COD (sCOD) was obtained using the same approach as that of tCOD measurement, except that the samples were initially passed through 1 µm filter paper prior to heating and analysis.

3.5.1.5 Inorganic nitrogen and phosphorous

The inorganic nitrogen and phosphorous concentration of wastewater and sludge (mixed liquor supernatant) were analysed. The samples were centrifuged (Beckman Coulter, USA) at 3728x*g* and 25 ºC for 10 min to remove large solids, and the resulting supernatant was filtered using 1 µm filter paper. The ammonia and phosphate concentration of the filtered supernatant were measured using flow injection analysis (Lachat Instruments, USA) following the Standard Method 4500 (Eaton *et al.*, 2005). Ammonia analysis involved the reaction of ammonia with phenol and hypochlorite to form a blue complex whose colour was intensified by nitroferricyanide, followed by measurement of the absorbance at 630 nm (Standard Method 4500-N) (Eaton *et al.*, 2005). Orthophosphate analysis involved the reaction of orthophosphate with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex that is reduced by ascorbic acid, followed by measurement of the absorbance at 880 nm (Standard Method 4500-P) (Eaton *et al.*, 2005). Meanwhile, the nitrite and nitrate concentration of filtered samples were measured using ion chromatography (Shimadzu, Japan) with Ionpac AS23 anion-exchange column.

3.5.1.6 Total phosphorous

The total phosphorous (TP) of wastewater was measured by first digesting 50 mL of sample with sulphuric acid solution for 2 h according to Standard Method 4500 (Eaton *et al.*, 2005), which converts organic phosphorous into orthophosphate. The digested sample was diluted to 100 mL using Milli-Q water and neutralised using 1 M of NaOH with phenolphthalein as indicator. The orthophosphate concentration of the diluted sample was analysed using flow injection analysis (Section 3.5.1.5).

3.5.1.7 Soluble microbial products and extracellular polymeric substances

Soluble microbial products (SMP) was extracted by centrifuging (Beckman Coulter, USA) sludge at 3,267x*g* at 4 °C for 15 min followed by filtration of supernatant with 0.45 µm

membrane to ensure removal of suspended solids. EPS was extracted from the same sample by resuspending the pellet in 10 mM NaCl adjusted to pH 10.5 using 1 M of NaOH. The resuspended mixture was purged with N_2 gas, immediately sealed off, and then shaken at 100 RPM at 25 °C. The solution was centrifuged (Beckman Coulter, USA) at 3267x*g* and 4 ºC for 15 min, and then filtered using 0.45 μm membrane to remove suspended solids and obtain EPS extract (Chon et al., 2011a). Proteins and carbohydrates were analysed using the modified Lowry method and phenol-sulphuric method, respectively (Hai et al., 2011; Wijekoon et al. 2013).

3.5.1.8 Total iron

To determine the concentration of total iron in sludge, samples were digested according to US EPA Method 3050b that involved digestion using nitric acid and hydrogen peroxide followed by addition of hydrochloric acid (Peña-Icart et al., 2011). The iron concentration of digested samples were measured using inductively-coupled plasma mass spectrophotometry (Agilent 7500CS, Agilent Technologies, USA).

3.5.1.9 Dissolved oxygen concentration, pH, and oxidation-reduction potential

The DO concentration of sludge was measured using a DO meter (YSI, USA). The pH and ORP of wastewater and sludge were measured using a pH/ORP meter (TPS, Australia).

3.5.2 Analysis of dewatering properties

To assess the effect of OSA on sludge dewaterability, two different techniques were used. First, the capillary suction time (CST) of unconditioned sludge samples from the control and OSA systems were determined. CST was measured by placing 5 mL of the sample in Type 304M CST meter (Triton Electronics Limited, UK). CST was the time (s) taken by water to permeate through a specific interval in a standard filter paper. The time was monitored using two electrodes that detected the water front. To eliminate the effect of solids concentration on filtration, the specific CST was obtained by dividing CST by the MLSS of the sample. Second, the dewatered cake TS concentration of WAS from the control ($WAS_{control}$) and OSA systems (WAS_{OSA}) were also determined using a previously described by To *et al.*, (2016). $WAS_{control}$ was the sludge discharged from $SBR_{control}$, whereas WAS_{OSA} was the sludge discharged from the external aerobic/anoxic reactor of OSA (Figure 3.1a), therefore, comparing these two parameters helped determining the impact of applying the OSA configuration on the WAS dewaterability.

WAS samples were conditioned by adding thickening polymer (Zetag8169, BASF, Australia) at the concentration of 7.5 g polymer/kg MLSS followed by manual stirring for five minutes. The conditioned sludge samples were placed on top of a filter paper (Whatman No. 4) secured inside a modified centrifuge tube, and then centrifuged (Beckman Coulter, USA) at 3728xg and 25 ºC for 15 min. The filterable fraction was forced through the filter paper and settled at the bottom of the centrifuge tube. The TS of the dewatered cake, which was the pellet scraped from the filter paper after centrifugation, was analysed as according to Standard Method 2540 (Eaton *et al.*, 2005).

3.5.3 Analysis of microbial community structure

3.5.3.1 DNA extraction and 16S rRNA gene amplicon sequencing

Sludge samples were collected from all reactors of the control and OSA systems in the fifth stage of the study (Table 3.1). Samples were stored and processed following the method described in Phan et al. (2016). Briefly, DNA extraction was carried out using the FastDNA @ spin kit for soil (MP Biomedical, New South Wales, Australia). DNA integrity and quality were assessed using 1% agarose gel electrophoresis and Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Extracted genomic DNA was sent to the Australian Genome Research Facility (Brisbane, Queensland, Australia) for sequencing. The V3-V4 regions of the 16S rRNA gene were amplified using primer pairs: 341F (5'–CTAYGGGRBGCASCAG–3') and 806R (5'–GGACTACNNGGGTATCTAAT–3'). Amplicon sequencing was conducted on the Illumina MiSeq platform, utilizing Illumina's Nextera XT Index's and Paired End sequencing chemistry. All sequencing data in this study are available at the Sequence Read Archive (SRP078298) in the National Centre for Biotechnology Information.

3.5.3.2 Sequence analyses

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.8). Primers were removed using Septk (version 1.2). The sequences were then processed using QIIME (version 1.9.1) (Caporaso *et al.*, 2010b) and USEARCH (version 8.1.1861) (Edgar, 2013) software packages. Following UPARSE pipeline, sequences were strictly filtered with maximum error rate of 0.5 and then trimmed to 240 bases. Full length

duplicates were discarded and sorted by abundance. Singletons were removed from the data set. Sequences were clustered followed by chimera filtering using "rdp_gold" database as reference. Reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned by *uclust* (Edgar, 2010) using Silva119 database (Pruesse *et al.*, 2007) in QIIME. Representative sequences were aligned using PyNAST (Caporaso *et al.*, 2010a) followed by gap filtering and then used to build phylogeny tree by FastTree (Price *et al.*, 2010).

The α- and β-diversities were measured at even sequencing depth of 50000 sequences per sample (minimum number of sequences found among samples). α-diversity indexes include observed species, Chao1, phylogenetic diversity (PD_whole_tree) and Shannon. The completeness of sampling was estimated by Good's coverage. For β-diversity comparison, an unweighted UniFrac distance (Lozupone and Knight, 2005) was calculated and then interpreted via PCoA (Principal Coordinate Analysis). All analyses were implemented in QIIME.

To explain phylogenetic variation of samples, constrained analysis of principal coordinates (CAP) (Anderson and Willis, 2003) and permutational multivariate analysis of variance using distance matrices (Adonis) were carried out. CAP uses a linear model combining several environmental variables (*i.e.* redox condition, SRT, and sludge interchange between aerobic and anoxic reactors) to predict the unweighted UniFrac coordinates. The significance of the factors in CAP model was ascertained using analysis of variance (ANOVA). Adonis with 999 permutations was used to supplement tests for significant differences in the community structure between redox, SRT and treatment conditions. The analysis was conducted using *phyloseq* (McMurdie and Holmes, 2013) and *vegan* packages (v2.3-5) (Oksanen *et al.*, 2013) in the *R* environment *(http://www.r-project.org/)*.

3.5.4 Analysis of TrOCs

3.5.4.1 Sample preparation

Duplicate wastewater and sludge samples were collected from all reactors of the control and OSA systems in the fourth stage of the study. All samples initially centrifuged (Beckman Coulter, USA) at 3728x*g* and 25 ºC for 10 min. To obtain TrOC concentration in the aqueous phase, the supernatant (influent, effluent, and sludge samples) was diluted to 500 mL in MilliQ

water, and then sequentially filtered using 1 µm and 0.7 µm glass fibre filter papers. These samples later underwent solid phase extraction (SPE).

To obtain TrOC concentration in the solid phase, the pellet (sludge samples only) was freeze-dried (Christ GmbH, Germany) for 12 h. The dried sample was ground to powder using mortar and pestle, and then 0.5 g of powder was placed in a capped glass vial. In the first round of extraction, the powder was re-suspended in 10 mL of methanol, vortexed (Ratek, Australia), and then ultrasonicated (Kleentek, Australia) for 10 min at 40 ºC. The mixture was centrifuged at centrifuged (Beckman Coulter, USA) at 3728x*g* and 25 ºC for 10 min, and then the supernatant was decanted and set aside. In the second round of extraction, the pellet from the previous extraction was re-suspended in 10 mL of dichloromethane and ethanol mixture (1:1 v/v), vortexed (Ratek, Australia), and then ultrasonicated (Kleentek, Australia) for 10 min at 40 ºC. The mixture was centrifuged (Beckman Coulter, USA) at 3728x*g* and 25 ºC for 10 min, and then the supernatant was decanted and added to the previous extract. The combined extract was diluted to 500 mL, and then sequentially filtered using 1 µm and 0.7 µm glass fibre filter papers. These samples later underwent SPE.

3.5.4.2 Solid phase extraction

Prior to SPE, the samples were spiked with 50 µL surrogate solution containing isotopically labelled standards (the list of compounds are provided in Chapter 8, Section 8.3.3.2) and mixed thoroughly. Surrogates were added to determine sample recovery. Then, the spiked samples were loaded to hydrophilic/lipophilic Oasis HLB cartridges (Waters, USA) that have been sequentially conditioned with 5 mL of methyl-tert-butyl ether, 5 mL of methanol, and twice with 5 mL of Milli-Q water. The loading rate (15 mL/min) was controlled by adjusting the vacuum pressure in the SPE manifold. After loading, the cartridges were rinsed with 5 mL of MilliQ, gently dried using N_2 gas, and then stored in a sealed bag at 4^oC until elution and analysis.

3.5.4.3 High performance liquid chromatography and mass spectrometry

The list of TrOCs that were analysed, along with their chemical properties and detection limits, are listed in Chapter 8, Section 8.3.3.2. TrOC concentration was measured using high performance liquid chromatography (HPLC, Agilent 1200, USA) coupled with tandem triple

quadrupole mass spectrometry (TQMS, Agilent 7000B, USA) as described in (McDonald *et al.*, 2012).

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CHAPTER 4: Effects of iron salt dosage on sludge reduction in the oxic-settling-anoxic (OSA) process

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4.1 INTRODUCTION

Despite several full-scale OSA installations (Troiani *et al.*, 2011; Coma *et al.*, 2013), there remains some contention regarding the mechanism/s responsible for sludge reduction in OSA. Chon *et al.* (2011a) hypothesised that oxygen- and substrate-deficient conditions in the external reactor enhance the disintegration of EPS, which are proteins, carbohydrates, and other biomolecules that serve as the structural framework of sludge flocs. Other researchers proposed that OSA enables 'metabolic uncoupling' and forces microorganisms to select energy replenishment over cellular propagation (Chudoba *et al.*, 1992), or that it transforms the ecology of activated sludge such that slow-growing bacteria or bacteriovores are enriched (Ye *et al.*, 2008). However, none of these mechanisms have been validated through investigations conducted with real wastewater. Furthermore, the maximum sludge reduction achieved by full-scale OSA (*e.g.*, 18%) (Troiani et al., 2011; Coma et al., 2013) was significantly lower than those of laboratory-scale implementations (*e.g.*, 58%) fed with synthetic wastewater (Chudoba *et al.*, 1992; Saby *et al.*, 2003b; Chon *et al.*, 2011b), which warrants further investigation using real wastewater.

The role of iron in the flocculation (Higgins and Novak, 1997) and floc destruction under anaerobic conditions (Novak *et al.*, 2003) has been reported, but its impact on OSA performance has not been systematically studied. Iron salts are commonly added to wastewater in full-scale plants for phosphorous removal by chemical process (Paul *et al.*, 2001; An *et al.*, 2014). When Fe(II) salt is added to an aerobic reactor, iron is spontaneously oxidised to Fe(III) given the availability of oxygen in the system (*i.e.*, $2Fe^{2+} + 2H^{+} + ½O_2 \rightarrow 2Fe^{3+} + 2H_2O$, $E°_{cell} = +2.0$ V) (Niu et al., 2013). Fe(III) forms hydroxyl complexes that serve as "ion bridge" between negatively-charged sites of EPS and causes flocculation (Higgins and Novak, 1997). The binding of Fe(III) to EPS appears to make sludge flocs less easily dispersed or destroyed. For example, Niu *et al.* (2013) observed that the addition of $FeCl_3$ (5-10 g/g DS) prevented the destruction of flocs by shear stress. Mishima and Nakajima (2009) also observed that the addition of $FeCl_3$ (2-5 g/L) decreased the release of EPS into the supernatant of a membrane bioreactor.

The ORP of the external reactor of OSA is a key parameter that impacts sludge reduction. Saby *et al.* (2003) observed that decreasing the ORP of the external reactor from +100 mV to less than -250 mV increased sludge reduction in a laboratory-scale OSA from 23 to 58%. However, very

low ORP levels are difficult to achieve under specific operational constraints, *e.g.*, at low HRT (Saby *et al.*, 2003b; Troiani *et al.*, 2011). Troiani *et al.* (2011) showed that treating sludge in alternating anaerobic (ORP= −400 to −200 mV) and anoxic ranges (ORP = −200 to +50 mV) in a full-scale plant resulted in sludge reduction of 13-17%. In a full-scale plant, maintaining an ORP range is more practical than trying to maintain a specific ORP value. In this context, an OSA containing both aerobic and anoxic stages in the external reactor rather than a strictly anaerobic reactor may be additionally beneficial in terms of minimising the influx of sCOD and nutrients in the main bioreactor upon recirculation of treated sludge. Thus it is worthwhile to investigate the performance of an OSA containing both aerobic and anoxic stages in the external reactor, which has not been reported in literature.

This objective of this chapter is to determine sludge reduction of an OSA system consisting of external aerobic/anoxic and anoxic reactors attached with an SBR receiving real wastewater, and to determine the impact of $FeCl_2$ addition on OSA performance. Preliminary batch tests were performed to investigate the effect of $FeCl_2$ addition on volatile solids reduction under alternating redox conditions. Then, the effect of $FeCl_2$ dosing on sludge reduction by the OSA system was assessed relative to a control system consisting of an SBR attached to a single-pass aerobic digester. The use of real wastewater is critical in that although real wastewater can undergo significant temporal variations, it produces more realistic biomass growth rates and sludge properties. The sludge yield, volatile solids reduction, and EPS concentrations of the reactors were monitored.

4.2 HYPOTHESIS

- The sludge yield of OSA may be lower than that of the control under specific conditions (*e.g.*, without $FeCl_2$ addition to domestic sewage).
- The addition of $FeCl_2$ may hamper sludge reduction in OSA.
- The disintegration of EPS is potentially an important mechanism in sludge reduction in OSA.

4.3 MATERIALS AND METHODS

This chapter involves batch (to be described in Section 4.3.1) and continuous (to be described in Section 4.3.2) experiments. The batch experiments were performed to determine the impact of $FeCl_2$ addition on EPS of sludge under aerobic/anoxic and anoxic conditions, which are

prevalent redox regimes that will be investigated in this chapter. Synthetic wastewater was used in batch experiments to strictly control the substrate and internal reactor conditions. Meanwhile, the continuous experiments were performed to determine the impact of $FeCl_2$ addition on sludge reduction in the OSA process. Domestic sewage was used as the influent (*i.e.*, feed to the SBRs) to cultivate sludge with realistic growth rate and properties and to avoid over-estimation of OSA performance.

4.3.1 Batch experiments

The batch reactors were inoculated with activated sludge from the aerobic reactor of Wollongong WWTP. The sludge was centrifuged (Beckman Coulter, USA) at 3728x*g* and 25 ºC for 10 min, and then re-constituted in synthetic wastewater to make up a total volume of 2 l. Synthetic wastewater (to be described in Section 4.3.3.1) was used only in the batch experiments to study the impact of $FeCl_2$ on volatile solids reduction.

Two redox regimes were implemented: aerobic/anoxic and anoxic. The batch aerobic/anoxic reactors with and without a single addition of 30 mg/L $FeCl_2$ were aerated in intermittent mode (*e.g.*, 8/16 hours aeration on/off) using an air diffuser placed at the bottom of the tank. Batch anoxic reactors with and without a single addition of 30 mg/L of $FeCl_2$ were completely sealed with a silicone-lined cap equipped with a sampling port and a gas outlet port with an air trap to prevent air leakage. All of the reactors were kept in a 25 °C water bath and continuously mixed by a magnetic stirrer for 30 d. MLSS, MLVSS, SMP, and EPS of sludge were measured after two weeks of incubation, with three and five sampling events for the batch aerobic/anoxic and anoxic reactors, respectively. Duplicate measurements were performed at each sampling event.

4.3.2 Continuous experiments

The continuous experiments involved laboratory-scale OSA (SBR_{OSA} attached to external aerobic/anoxic and anoxic reactors) and control ($SBR_{control}$ attached to single-pass aerobic digester) systems with configurations that are described in Chapter 3 (Section 3.2).

Two SBRs were initially inoculated with aerobic activated sludge from Wollongong WWTP. They had HRT of 12 h and SRT of 10 d, and were fed with settled domestic sewage (to be described in Section 4.3.3.2). They were operated for 87 d with addition of 15 mg/L of $FeCl_2$ in

the influent tank starting from the 53rd day of operation. At the 88th day of operation, SBR_{OSA} was integrated with an external aerobic/anoxic reactor (2 L) and an anoxic reactor (2 L) to form the OSA system (Section 3.2.1) and $SBR_{control}$ was attached to a 2-L aerobic digester to form the control system (Section 3.2.2).

The detailed operation of the OSA system is described in Chapter 3 (Section 3.2.1). The SIR of the external reactors was maintained by transferring 16.5% of sludge from the anoxic to the aerobic/anoxic reactor (q_4) and 16.5% of sludge from the anoxic reactor to SBR_{OSA} (q_5). The SRT of the external reactors was maintained at 20 d. At steady-state, SBR_{OSA} had a pH of 6-8 and DO concentration of 4-5 mg/L. The aerobic/anoxic reactor had a pH of 5-7, DO concentration of less than 1 mg/L, and ORP of +50 to +100 mV (measurements obtained when aeration was off). The anoxic reactor had an ORP range of −400 to −300 mV.

The detailed operation of the control system is described in Chapter 3 (Section 3.2.2). At steady-state, $SBR_{control}$ had a pH of 6-8 and DO concentration of 4-5 mg/L. The aerobic digester had a pH of 5-7, DO concentration of 4-5 mg/L, and ORP of +180 to +340 mV.

To study the effect of $FeCl_2$ on OSA, $FeCl_2$ dosing was halted on the 152nd day of operation and then resumed at 30 mg/L on the 196th day of operation. A summary of the experimental phases in this chapter is shown in Table 4.1. It is noteworthy that the background total iron concentration in the wastewater was 1.52±0.68 mg/L (n=12). Thus the influent to the SBRs was not completely devoid of iron even though it was not supplemented with $FeCl_2$.

Table 4.1. Summary of the experimental phases of the continuous reactor operation in this chapter. $FeCl_2$ dosage to the influent was varied (0-30 mg/L) while SRT_{SBR} was maintained at 10 d, SRT_{ext} was maintained at 20 d, and the SIR of OSA was maintained at 16.5%.

Experimental phase	Operation period (d)	$FeCl_2$ dosage (mg/L)
I [a]	88	15 [b]
II	63	15
III	43	None
IV	43	30

[a] Start-up phase
[b] Dosing was began on the 53rd day of operation

4.3.3 Wastewater

4.3.3.1 Synthetic wastewater

The synthetic wastewater used for batch experiments was composed of glucose (400 mg/L), peptone (100 mg/L), urea (35 mg/L), monopotassium phosphate (17.5 mg/L), magnesium sulphate (17.5 mg/L), ferrous (10 mg/L), and sodium acetate (225 mg/L).

4.3.3.2 Domestic sewage

Unsettled domestic sewage (Table 4.2) used for continuous experiments was obtained from the outlet of the primary sedimentation tank of Wollongong WWTP. It was collected weekly and stored at 4 °C until use.

Table 4.2. Properties of unsettled domestic sewage where *n*=number of samples

Property	**Average**	***n***
sCOD	63±30 mg/L	38
TOC	43.1±21.2 mg/L	40
TN	35.2±4.1 mg/L	40
NH_4^+-N	18.8±6.1 mg/L	38
PO_4^{3-}-P	17.1±11.2 mg/L	38
Total P	21.3±14.5 mg/L	28
pH	7.7±4.7	31

4.3.4 Calculation of sludge reduction

Sludge reduction was calculated as the difference in sludge yield of SBR_{OSA} and $SBR_{control}$. In the current study, sludge yield *P* is defined as slope of the linear regression of cumulative sludge produced in terms of MLVSS (*P*) over the cumulative substrate consumed in terms of sCOD (*C*). The calculation of sludge yield is detailed in Chapter 3 (Section 3.4).

The reduction in the volatile solids fraction of sludge was also assessed by calculating the change in MLVSS/MLSS ratio:

$$MLVSS/MLSS\ reduction\ (\%) = \frac{MLVSS_0/MLSS_0 - MLVSS_i/MLVSS_i}{MLVSS_0/MLSS_0} \times 100 \qquad \textbf{Equation 4.1}$$

where $MLVSS_0/MLSS_0$ is the initial ratio and $MLVSS_i/MLSS_i$ is the ratio of at any given time *i*.

4.3.5 Analytical techniques

4.3.5.1 Wastewater and sludge properties

The solids concentration and SVI of sludge were measured as described in Chapter 3 (Section 3.5.1.1 and 3.5.1.2, respectively). The solids concentration, TOC/TN, sCOD concentration, ammonia concentration, and phosphate concentration of wastewater were measured as described in Chapter 3 (Section 3.5.1.1-3.5.1.5). The DO concentration, pH, and ORP of wastewater and sludge were measured as described in Chapter 3 (Section 3.5.1.9).

4.3.5.2 Total phosphorous

The TP of wastewater was measured as described in Chapter 3 (Section 3.5.1.6).

4.3.5.3 *Soluble microbial products and extracellular polymeric substances*

The SMP and EPS concentration of sludge was measured as described in Chapter 3 (Section 3.5.1.7). Two-sample *t*-test was performed using Analysis Toolpak in Microsoft Excel to determine if there was significant difference in the EPS concentrations of different sets of samples. $p < 0.05$ was considered to indicate statistical significance.

4.3.5.4 Total iron

The total iron concentration of sludge was measured as described in Chapter 3 (Section 3.5.1.8).

4.4 RESULTS AND DISCUSSION

4.4.1 Batch experiments: impact of $FeCl_2$ addition on sludge biodegradation under different redox regimes

$FeCl_2$ addition to the batch aerobic/anoxic reactor increased $EPS_{protein}$ concentration (Two sample *t*-test; $t(6)=0.91$, $p=0.41$) and decreased MLVSS reduction (Table 4.3). This was because Fe(II) was oxidised to Fe(III) in this reactor due to its relatively high ORP (+30 to +80 mV) (Niu *et al.*, 2013), and Fe(III) reacted with the biopolymers in sludge flocs and hindered the disintegration of the EPS (Niu *et al.*, 2013). Notably, variations in EPS and SMP concentrations were primarily observed in the protein fraction due to the preferential binding of Fe(III) to proteins (Novak *et al.*, 2003). On the contrary, $FeCl_2$ addition to the batch anoxic reactor (ORP = –400 to –300 mV) decreased $EPS_{protein}$ and enhanced MLVSS reduction (Table 4.3).

Table 4.3. MLVSS/MLSS reduction and average EPS and SMP of the batch reactors. The values are the average ± standard deviation where *n* = number of measurements.

Batch reactor	**MLVSS/MLSS reduction [a] (%)**	**EPS [b]**		**SMP [b]**		***n***
		Protein (mg/g MLVSS)	*Carbohydrate (mg/g MLVSS)*	*Protein (mg/L)*	*Carbohydrate (mg/L)*	
Aerobic/anoxic	25	5.4±3.6	1.0±0.6	9.8±1.6	6.3±2.8	5
Aerobic/anoxic +$FeCl_2$	18	10.1±2.9	1.3±0.4	7.7±0.7	14.0±6.3	5
Anoxic	24	21.1±15.9	7.3±4.9	16.7±5.8	18.6±12.2	3
Anoxic +$FeCl_2$	29	12.8±8.7	6.1±5.1	30±11.3	21.5±17.4	3

[a] MLVSS/MLSS reduction calculated at Day 30 of incubation
[b] Average of measurements obtained from Day 14 to 30

Fe(III) can lead to sludge flocculation due to ion bridging and surface charge neutralisation (Higgins and Novak, 1997). In the flocculation process, the outer EPS layer called the "loosely-bound EPS" and the inner EPS layer called the "tightly-bound EPS" are both compressed as flocs aggregate (Niu *et al.*, 2013). Studies report that Fe(III) strongly retains biopolymers within flocs (Murthy and Novak, 2001), and decreases the extractability of the loosely-bound EPS (Niu *et al.*, 2013). However, during anaerobic respiration, Fe(III) can be converted to Fe(II). This results in the release of EPS into solution, especially those in the form of proteins, and eventually to deflocculation (Novak *et al.*, 2003). Park *et al.* (2006) further suggests that the reduction of Fe(III) is a prerequisite to the destruction of volatile solids under anaerobic digestion. Thus, in

the current study, MLVSS reduction was facilitated in the batch anoxic reactor (ORP < -250 mV), and not in the batch aerobic/anoxic reactor (ORP = +30 to +80 mV) where bacterial population capable of Fe(III) reduction may not have been enriched. Indeed, in the presence of $FeCl_2$, the anoxic reactor showed nearly twice as much $EPS_{protein}$ in solution (*i.e.*, $SMP_{protein}$) than the aerobic/anoxic reactor (Table 4.3), confirming that Fe(III) reduction (and hence volatile solids reduction) was impaired in intermittently aerated (*i.e.*, aerobic/anoxic) conditions.

The batch reactor investigations systematically demonstrated that aerobic/anoxic treatment of sludge achieved similar MLVSS reduction as anoxic treatment in the absence of Fe(III). However, when significant (*e.g.*, at least 30 mg/L) Fe(III) were present in the sludge, aerobic/anoxic treatment did not effectively reduce volatile solids. These observations form an important baseline for an explanation of the results from OSA operation with real wastewater.

4.4.2 Continuous experiments: impact of $FeCl_2$ addition on the performance of continuous OSA fed with domestic sewage

4.4.2.1 Basic reactor performance and sludge properties

The influent had a wide range of COD (Figure 4.1) and nutrient concentration (Figure 4.2) due to temporal variation in domestic sewage. The two SBRs had comparable effluent COD concentration during the start-up phase (Figure 4.1). Attachment of the external aerobic/anoxic and anoxic reactors caused a temporary increase in the effluent COD of SBR_{OSA}, probably because the reactor received surplus COD from the returned sludge. Nonetheless, SBR_{OSA} quickly acclimatised and from then on, the effluent COD of $SBR_{control}$ and SBR_{OSA} were comparable (Figure 4.1). Similarly, the effluent TOC concentration $SBR_{control}$ and SBR_{OSA} were comparable and therefore the TOC removal efficiencies of the reactors were similar (Figure A.1). This indicates that OSA did not impact these parameters.

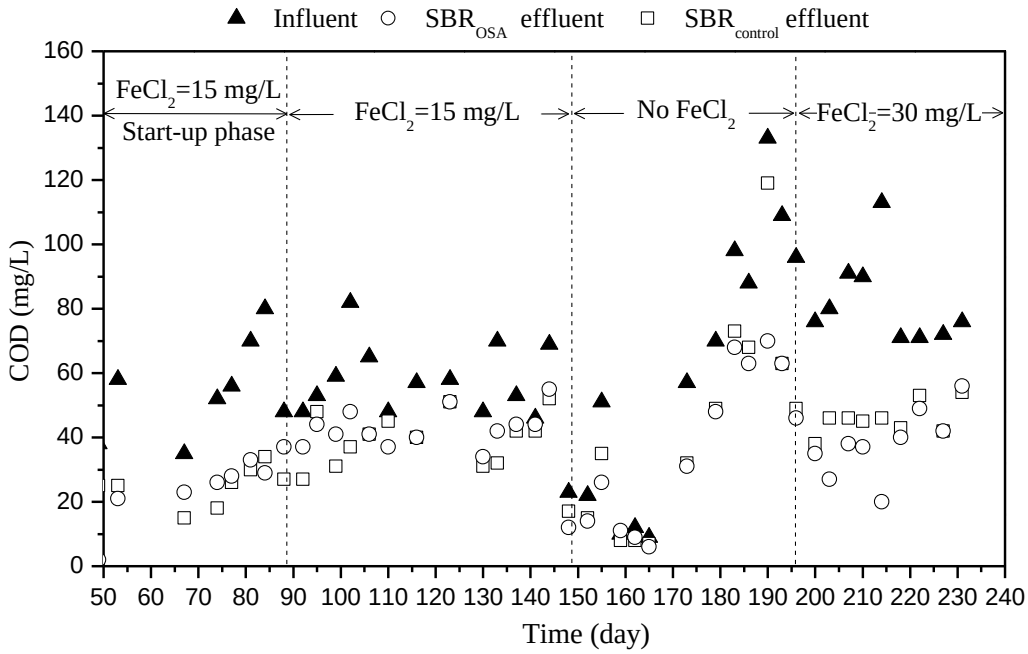

Figure 4.1. COD concentrations of SBR_{OSA} and $SBR_{control}$ at different dosages of $FeCl_2$ (0-30 mg/L) to the influent (settled domestic sewage). The dashed lines indicate change in $FeCl_2$ dosage.

$SBR_{control}$ and SBR_{OSA} had comparable effluent ammonia concentration throughout the period of operation (Figure 4.2). The effluent TN concentration of the SBRs was also comparable and therefore their TN removal efficiencies were similar (Figure A.2). Moreover, the SBRs had poor orthophosphate removal performance. $SBR_{control}$ and SBR_{OSA} had no orthophosphate removal when $FeCl_2$ dosage was 0-15 mg/L, and achieved only up to 30% orthophosphate removal when $FeCl_2$ was 30 mg/L (Figure 4.2). This was because the Fe/P molar ratios (*i.e.*, 1.27 and 0.85 for $FeCl_2$ dosage of 15 and 30 mg/L, respectively) were lower than the theoretical ratio required for chemical precipitation (*e.g.*, 1.5) (An *et al.*, 2014).

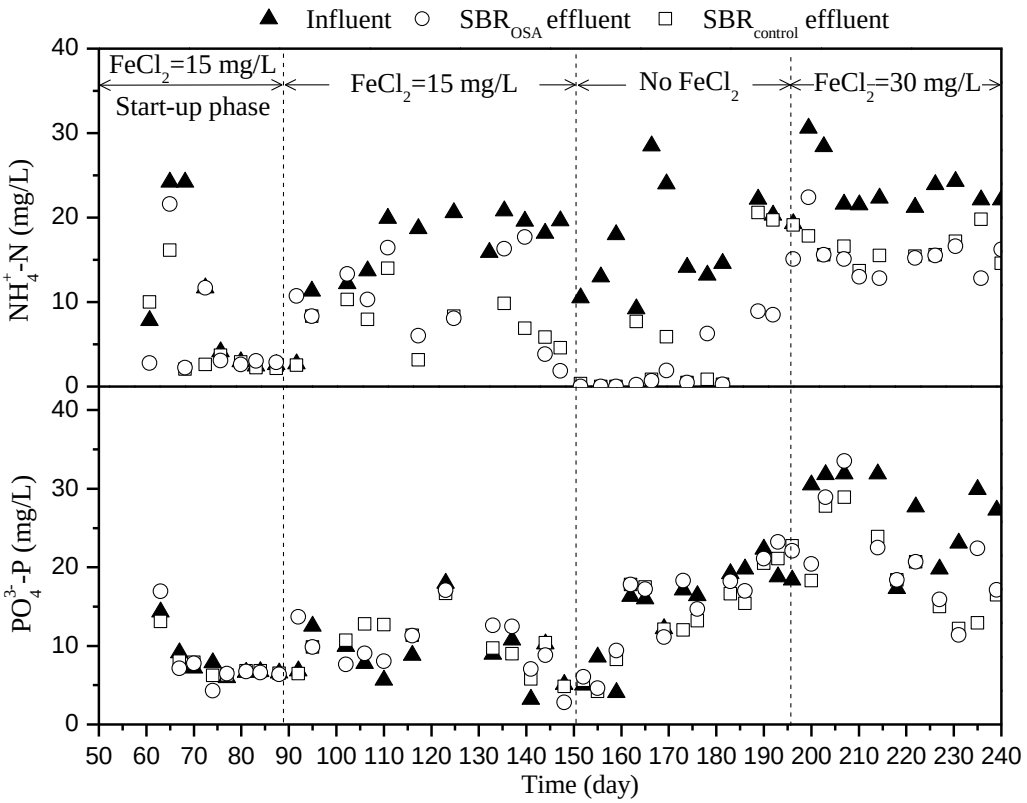

Figure 4.2. Ammonia and orthophosphate concentrations in $SBR_{control}$ and SBR_{OSA} effluent at different dosages of $FeCl_2$ (0-30 mg/L) to the influent (settled domestic sewage). The dashed lines indicate change in $FeCl_2$ dosage.

The SVI was below 100 ml/g for both SBRs irrespective of iron dosing (Figure 4.3). This indicates that the SBRs possessed rapidly settling flocs (Tchobanoglus et al., 2003), and that OSA did not improve sludge settleability.

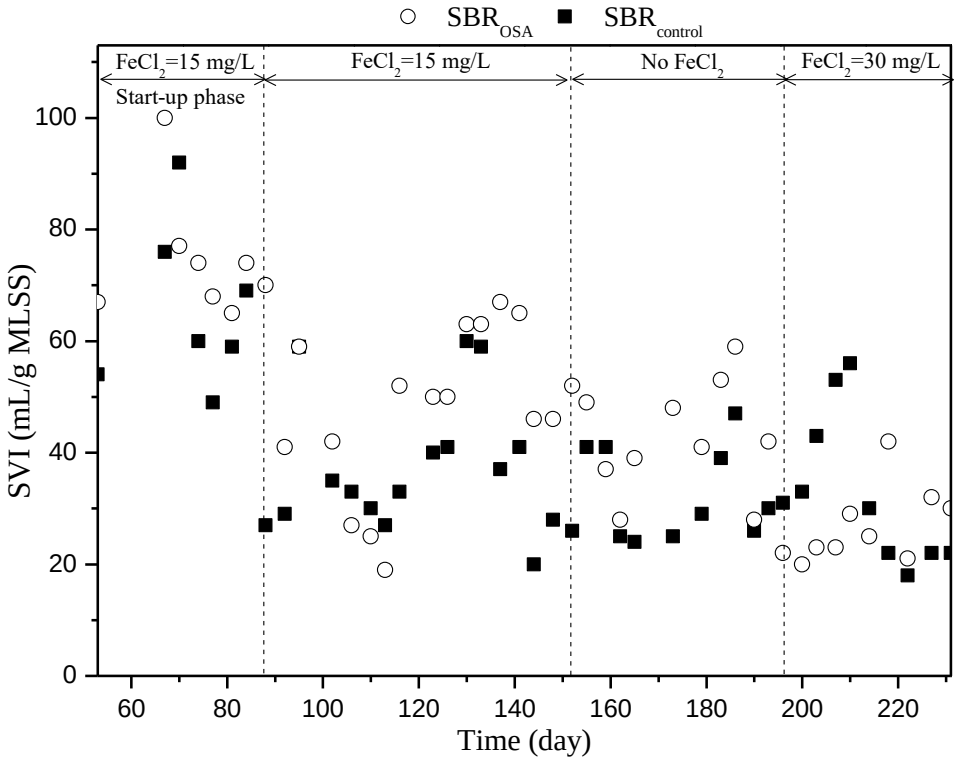

Figure 4.3. SVI of SBR_{OSA} and $SBR_{control}$ at different dosages of $FeCl_2$ (0-30 mg/L) to the influent (settled domestic sewage). The dashed lines indicate change in $FeCl_2$ dosage.

Like the current study, others also found that OSA had negligible impact on COD (Chudoba et al., 1992; Saby et al., 2003; Goel and Noguera, 2006) and TN removal (Ye et al., 2008) of the main aeration tank. However, an additional aspect revealed in the current study was that fluctuations in influent wastewater strength (sCOD=9-133 mg/L; *n*=41) similarly affected the COD (Figure 4.1) removal performance of the control SBR_{OSA} and $SBR_{control}$. This influent COD fluctuation was also observed to somewhat affect the volatile solids reduction capacity of the OSA system (data not shown) although the trend of volatile solids reduction discussed in Section 4.4.2.2 was consistent.

4.4.2.2 Impact of $FeCl_2$ addition on OSA performance

The impact of $FeCl_2$ addition on OSA performance in terms of sludge reduction was analysed. The sludge yield of SBR_{OSA} and $SBR_{control}$ (Table 4.4) was derived from the corresponding plots

of cumulative sludge produced versus cumulative substrate consumed in the SBRs (Figure 4.4). Because of the significant variation in the wastewater strength, comparing the sludge yield of a reactor across different runs did not give meaningful trends. Therefore, to eliminate interference from the varying influent, the effect of $FeCl_2$ dosage was observed by contrasting the sludge yield of SBR_{OSA} and $SBR_{control}$ at each experimental phase only (Table 4.4).

Table 4.4. Sludge yield of OSA and control at different dosages of $FeCl_2$ (0-30 mg/L) to the influent (settled domestic sewage).

Experimental phase	**$FeCl_2$ dosage (mg/L)**	**Sludge yield *Y* (g MLVSS/g sCOD)**							
		$SBR_{control}$	R^2	*Control system* [b]	R^2	SBR_{OSA}	R^2	*OSA system* [c]	R^2
I	15	3.61	0.94	-	-	3.51	0.98	-	-
II	15	4.29	0.91	3.90	0.73	7.62	0.87	9.72	0.85
III	None	10.54	0.85	8.75	0.87	7.87	0.93	6.69	0.96
IV	30	1.47	0.77	1.14	0.66	2.67	0.97	2.74	0.95

[a] Before attaching the external reactors to the SBRs
[b] Control system consisted of $SBR_{control}$ and aerobic digester
[c] OSA system consisted of SBR_{OSA} and external aerobic/anoxic and anoxic reactors

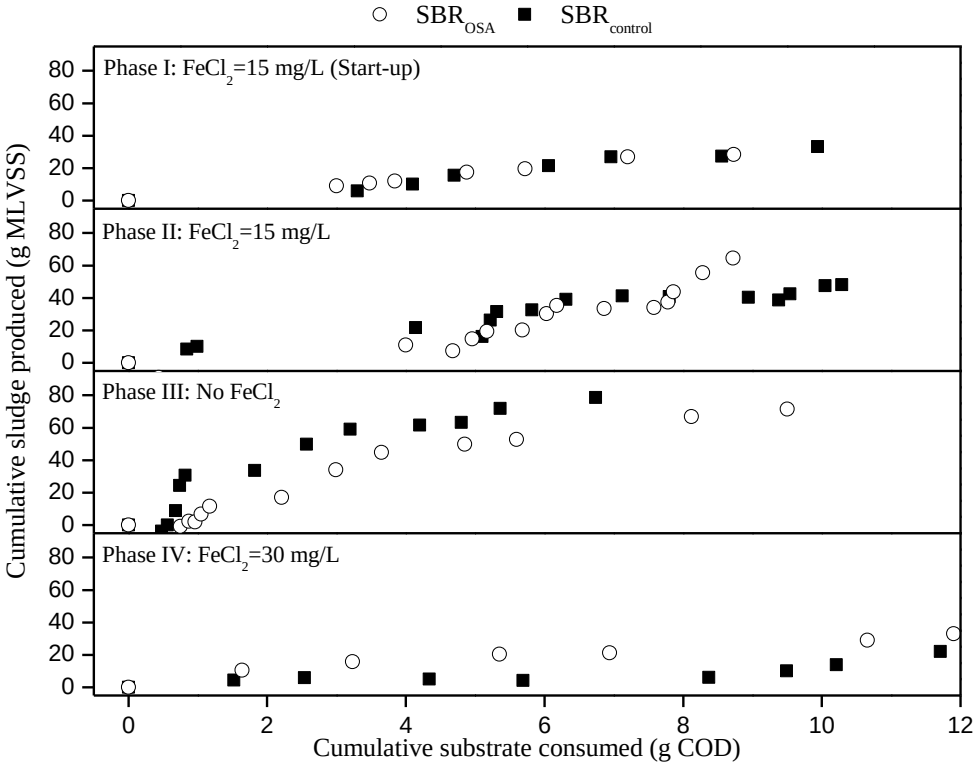

Figure 4.4. Cumulative sludge produced (g MLVSS) versus cumulative substrate consumed (g sCOD) of SBR_{OSA} and $SBR_{control}$ at different dosages of $FeCl_2$ (0-30 mg/L) to the influent (settled domestic sewage).

SBR_{OSA} and $SBR_{control}$ had similar sludge yield during the start-up phase (Table 4.4). In other words, the SBRs equally acclimatised to wastewater characteristics and operation conditions and the experiments had similar initial conditions.

When 15 and 30 mg/L of $FeCl_2$ were added to the influent, the sludge yield of SBR_{OSA} was higher than that of the control $SBR_{control}$ (Table 4.4), meaning that the OSA process was unable to reduce the MLVSS production. The sludge yield of the OSA system (*i.e.*, SBR_{OSA}+external reactors) was also greater than that of SBR_{OSA} (Table 4.4, derived from Figure A.3), which could indicate that the external reactors had a net MLVSS production. This is supported by the fact that the MLVSS/MLSS reduction of the external reactors was mostly in negative values (Figure 4.5). On the contrary, without $FeCl_2$ addition, the Y_{obs} of SBR_{OSA} was lower than that of the control $SBR_{control}$ by 24.8% (Table 4.4), evidencing that OSA reduced the MLVSS production. Furthermore, without $FeCl_2$ dosing, (i) the Y_{obs} of the entire OSA system was lower than that of SBR_{OSA}, and (ii) the MLVSS/MLSS ratio of sludge fed to the aerobic/anoxic reactor was reduced (Figure 4.5).

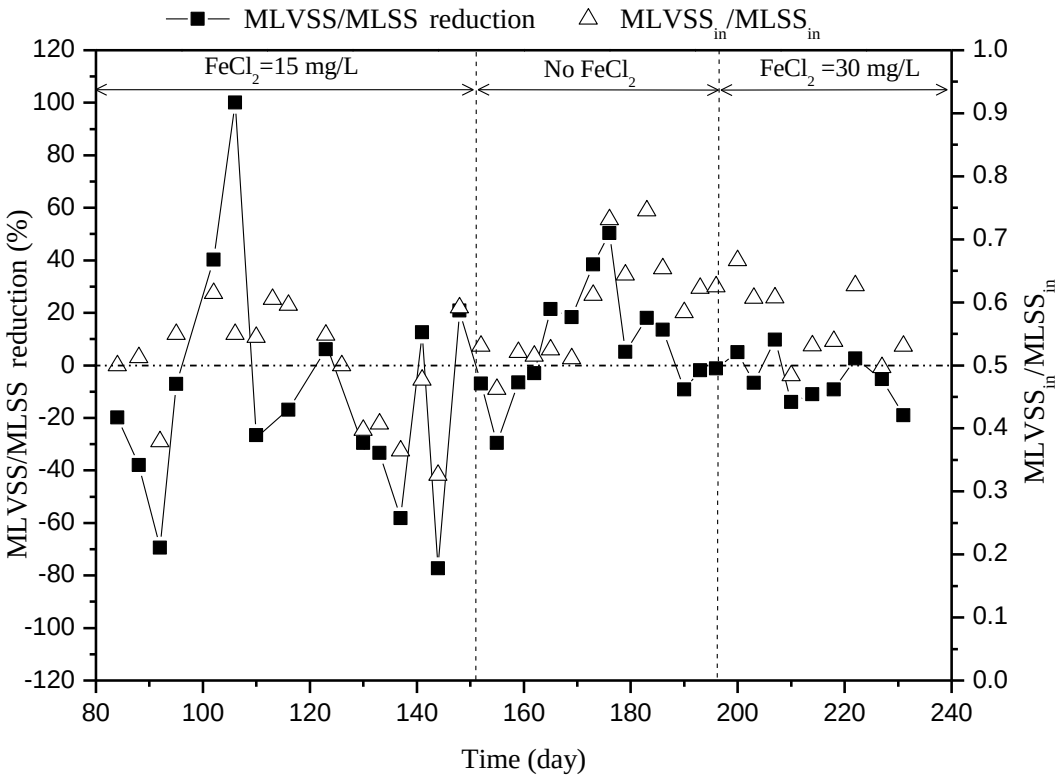

Figure 4.5. Reduction (%) of MLVSS/MLSS ratio achieved by the external reactors superimposed with the $MLVSS_{in}/MLSS_{in}$, the ratio of the thickened sludge fed to the aerobic/anoxic reactor at different dosages of $FeCl_2$ (0-30 mg/L) to the influent (settled domestic sewage).

To understand the impact of $FeCl_2$ addition on OSA performance, the SMP and EPS profiles of the reactors were investigated (Figure 4.6). The difference in the EPS profiles of $SBR_{control}$ and SBR_{OSA} was not ascertained due to the significant variability of data points in each experimental run (Figure A.4). A significant increase in the $EPS_{protein}$ of the external aerobic/anoxic reactor (Figure 4.6a) occurred when $FeCl_2$ concentration was changed from zero (33.2±9.8 mg/g; n=4) to 30 mg/L (55.7±10.8 mg/g; n=5) (Two sample t-test; $t(7)=3.57$, $p=0.014$). Correspondingly, the $SMP_{protein}$ (Figure 4.6) and $SMP_{carbohydrate}$ (Figure A.5) of the aerobic/anoxic reactor decreased. These findings suggest that $FeCl_2$ dosing reduced the disintegration of EPS especially in the aerobic/anoxic reactor, and consequently decreased the efficiency of OSA to degrade MLVSS. The deleterious effect of $FeCl_2$ dosing on EPS disintegration and sludge reduction was also observed in the batch aerobic/anoxic reactors (Section 4.4.1), and was possibly due to the inefficiency of aerobic/anoxic conditions to biologically reduce Fe(III) that bound EPS. MLVSS reduction in the batch aerobic/anoxic reactor was greater than that of its continuous counterpart

probably because the former was fed with synthetic wastewater and thus under ideal conditions. Nonetheless, both batch and continuous reactors showed that Fe(III) prevented EPS degradation under aerobic/anoxic conditions.

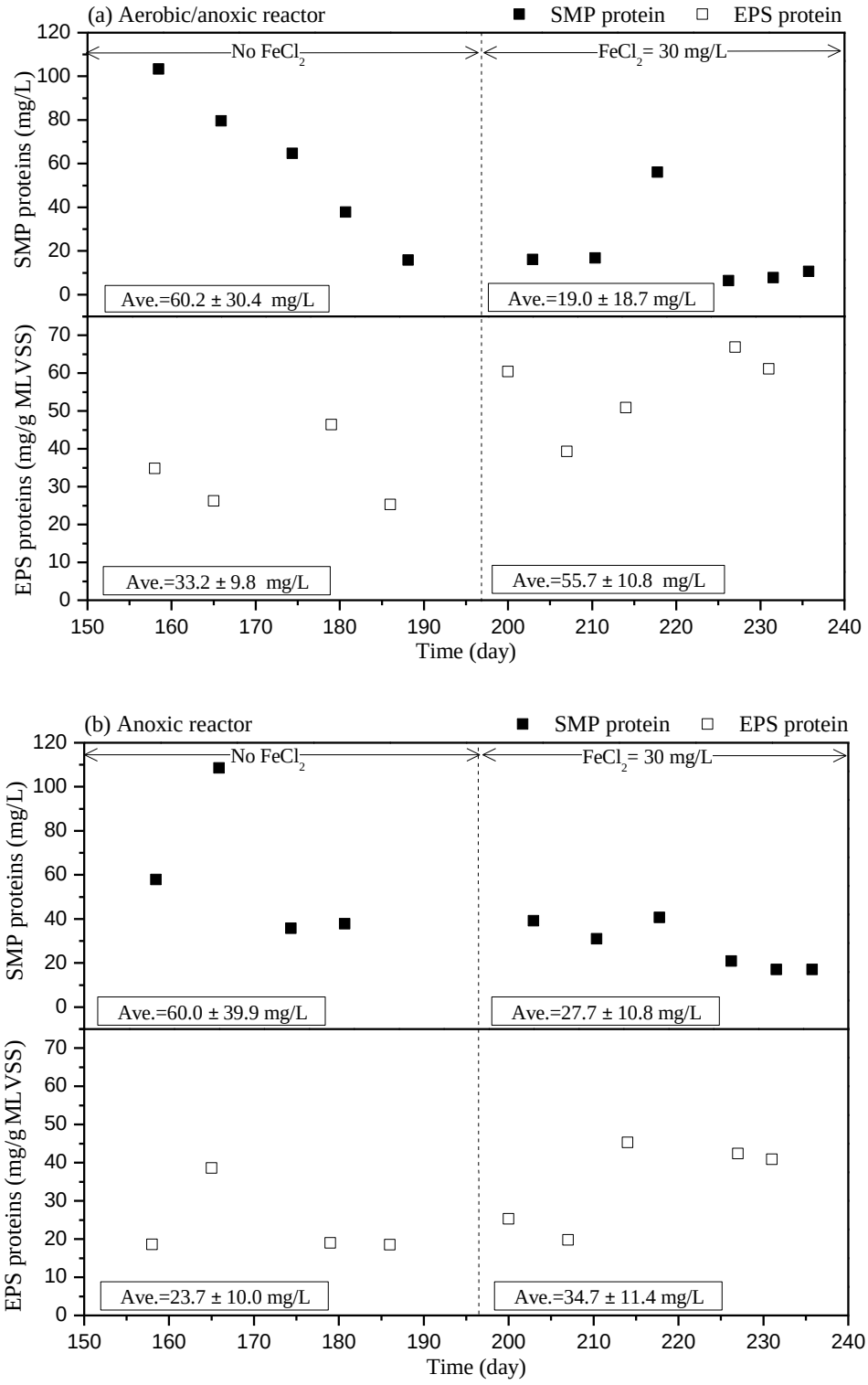

Figure 4.6. SMP and iron-associated EPS in the form of proteins of the (a) aerobic/anoxic and (b) anoxic reactors of OSA when FeCl2 dosage to the influent (unsettled domestic sewage) was zero (Phase III) and 30 mg/L (Phase IV).

Notably, the $EPS_{protein}$ of the anoxic reactor (Figure 4.6b) slightly increased when $FeCl_2$ concentration was changed from zero (23.7±10.0 mg/L; *n*=4) to 30 mg/L (34.7±11.4 mg/L; *n*=5), but the change was not statistically significant (Two sample *t*-test; $t(7)=1.55$, $p=0.17$). This indicates that EPS degradation in the anoxic reactor was not as impacted by $FeCl_2$ dosing as the aerobic/anoxic reactor. Nonetheless unlike the batch anoxic reactor (Section 3.1), the continuous anoxic reactor of OSA did not exhibit enhancement of EPS disintegration with $FeCl_2$ dosing. This was probably because the anoxic reactor received less destructible flocs from the aerobic/anoxic reactor, whereas the batch anaerobic reactor stood alone. Moreover, it had a much lower SRT (10 d) than that of the batch anoxic reactors, which was 490 d (calculated from the sludge spent for analysis).

4.4.2.3 Mechanisms of sludge reduction in OSA with dual-redox external reactors

The vulnerability of OSA to $FeCl_2$ dosing elucidates the critical role that the aerobic/anoxic reactor plays in this particular OSA configuration. The dual-redox external reactor that was utilised in the current study is distinct from the OSA configurations reported in literature, which commonly involves a single anoxic or anaerobic external tank (Saby *et al.*, 2003a; Goel and Noguera, 2006; Coma *et al.*, 2013). Anaerobic condition in OSA (*e.g.*, ORP = −250 V) has been found to favour sludge reduction (Saby *et al.*, 2003a). Nonetheless, the current study demonstrates that sludge reduction can also occur in intermittently aerated (*i.e.*, aerobic/anoxic) and anoxic conditions that may be easier to implement in full-scale operation (Troiani *et al.*, 2011). However, the volatile solids reduction capacity of this configuration, particularly that of the aerobic/anoxic reactor, is susceptible to iron dosing.

In the particular OSA configuration investigated in the current study, it is possible that the aerobic/anoxic reactor facilitated the hydrolysis of proteins, carbohdyrates, and other macromolecules, thereby enhancing subsequent degradation in the anoxic reactor. The aerobic/anoxic reactor could have also helped ensure that O_2, NO_3^- , and COD are depleted as much as possible so that the anoxic reactor was deficient of oxygen and substrate. Furthermore, the intermittent aeration in the aerobic/anoxic reactor possibly created alternating redox conditions that could trigger faster biodegradation.

The current findings confirm that without $FeCl_2$ dosing, the OSA process reduces sludge in two ways: (i) it decreases the MLVSS/MLSS of sludge fed to the external reactors (Figure 4.5), and (ii) it decreases the sludge yield of the main aeration tank (Table 4.4). The reduction in volatile solids content of waste sludge may have implications on its treatability and odour reduction during post-processing and transport. The influence exerted by OSA on the biomass growth in the main bioreactor has been reported in earlier studies (Chudoba *et al.*, 1992; Saby *et al.*, 2003a). For example, Chudoba *et al.* (1992) reported that alternating sludge between favourable and non-favourable growth conditions result in metabolic uncoupling in microorganisms, which forces the biomass that is returned to the main bioreactor to prioritise energy replenishment instead of cellular propagation. The current study provides compelling evidence of lower sludge production in the main bioreactor as a result of the OSA process.

4.4.2.4 Verification of the effect of $FeCl_2$ dosing on solids concentration analysis

A previous study showed that iron can precipitate as hydrated vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) that may cause over-estimation of MLVSS as it loses 17% of its weight upon incineration at 550 °C (Tien and Waugh, 1969). Vivianite has been observed in iron-amended anoxic reactors (Frossard *et al.*, 1997). Nonetheless, in the current study, the formation of vivianite in the continuous reactors was unlikely due to the insufficiency of iron in the influent. The average molar Fe/P ratio in the influent tank was only 1.27 and 0.85 at the period when $FeCl_2$ concentration was 15 (Phase II) and 30 mg/L (Phase IV), respectively (Table 4.5). An *et al.* (2014) investigated the formation of vivianite in synthetic wastewater with $FeCl_2$ dosing and found that the Fe/P molar ratio should be more than three to enable significant vivianite formation. The authors attributed this to the partial formation of ferrous hydroxides, which hindered the formation of vivianite.

Table 4.5. Orthophosphate concentration and Fe/P molar ratio of the influent (unsettled domestic sewage) at different phases of the experiment (*n* = number of measurements)

Experimental phase	$FeCl_2$ dosage (mg/L)	*n*	Minimum influent PO_4^{3-}-P (mg/L)	Maximum influent PO_4^{3-}-P (mg/L)	Average influent PO_4^{3-}-P concentration (mg/L)	PO_4^{3-}-P standard deviation (mg/L)	Minimum influent molar Fe/P	Maximum influent molar Fe/P	Average influent molar Fe/P
II	15	12	3.22	17.96	8.79	3.75	0.62	3.47	1.27
III	None	12	5.01	22.3	14.65	5.90	NA	NA	NA
IV	30	10	17.30	31.90	26.23	1.71	0.69	1.28	0.85

It was also observed that the total Fe concentration, *i.e.*, combined Fe(II) and Fe(III), of the sludge of SBR_{OSA} steadily increased when $FeCl_2$ dosage was increased from zero (Phase III) to 30 mg/L (Phase IV) due to the accumulation of metal precipitates, whereas that of $SBR_{control}$ increased and then decreased (Figure 4.7). The fluctuation in Fe concentration in $SBR_{control}$ was probably due to the wash out of solids. If vivianite had formed and caused over-estimation of MLVSS, the MLVSS/MLSS ratio should have increased when $FeCl_2$ dosage was increased from zero to 30 mg/L. However, it was observed that the MLVSS/MLSS ratio of SBR_{OSA} decreased and that of $SBR_{control}$ remained the same (Figure 4.7). This was because the accumulation of metal precipitates in the reactor increased MLSS as observed in other studies (Paul *et al.*, 2001; Li, 2005), but not MLVSS.

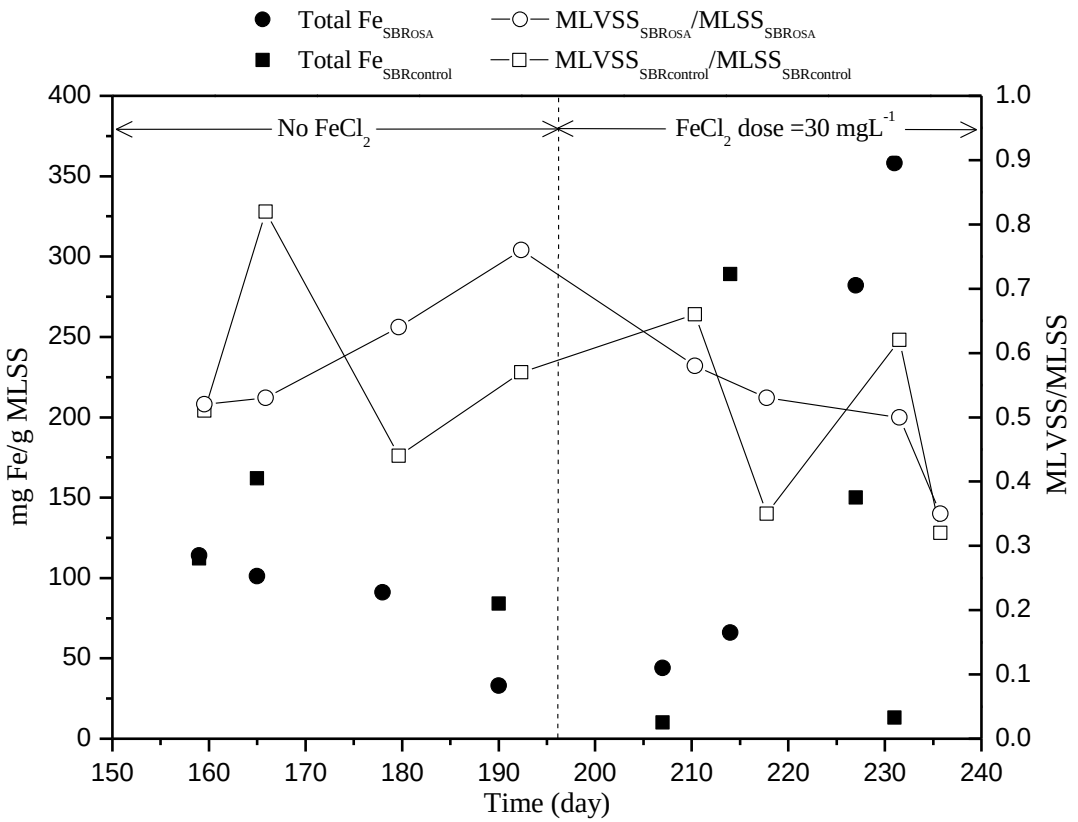

Figure 4.7. Total Fe concentration of the sludge superimposed with MLVSS/MLSS ratio of $SBR_{control}$ and SBR_{OSA} when $FeCl_2$ dosage to the influent (0-30 mg/L) was zero (Phase III) and 30 mg/L (Phase IV)

4.5 CONCLUSIONS

Based on investigations conducted with a continuous flow OSA system receiving real wastewater, it was demonstrated for the first time that the addition of $FeCl_2$ is counterproductive to sludge reduction in the external intermittently aerated (*i.e.*, aerobic/anoxic) reactor. Batch tests showed that $FeCl_2$ dosing decreased the volatile solids reduction of a batch aerobic/anoxic reactor probably due to a decline in the destructibility of EPS. This parallels the findings in continuous OSA operation, wherein it was found that the external aerobic/anoxic reactor had greater EPS and lower SMP when there was $FeCl_2$ dosing. In contrast, $FeCl_2$ did not have any negative effect on sludge reduction of the batch anoxic reactor, and had less severe impact on EPS destruction in the external anoxic reactor during continuous operation. This was probably because anoxic conditions facilitated the biological reduction of Fe(III) causing deflocculation and eventual sludge degradation. Without $FeCl_2$ addition, the sludge yield of the SBR_{OSA} was 24.8% lower than that of the $SBR_{control}$. Results reported here validate two mechanisms of sludge reduction (in absence of iron addition of more than 15 mg/L) by the OSA process: first, the external reactors reduce the volatile solids of waste activated sludge and second, the interchange of sludge decreases volatile solids production in the main bioreactor.

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CHAPTER 5: Effects of sludge interchange rate (SIR) on sludge reduction in the oxic-settling-anoxic (OSA) process

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5.1 INTRODUCTION

Laboratory-scale OSA fed with synthetic wastewater have shown promising sludge reduction (*e.g.*, more than 50%) (Saby *et al.*, 2003; Novak *et al.*, 2007; Sun *et al.*, 2010; Chon *et al.*, 2011). However, these high sludge reduction values have not been realised in full-scale systems (Troiani *et al.*, 2011; Coma *et al.*, 2013). In Chapter 4, 24.8% reduction in the sludge yield was achieved by a laboratory-scale OSA operated using real sewage if iron salt was not added to the influent (Semblante *et al.*, 2015). Other studies have demonstrated that OSA performance is influenced by different operation conditions, such as oxidation reduction potential (ORP), sludge retention time (SRT), and sludge loading rate of the external reactor (Saby *et al.*, 2003; Wei *et al.*, 2003; Ye *et al.*, 2008; Foladori *et al.*, 2010; Coma *et al.*, 2013). To date, the manipulation of these parameters has only resulted in variable and inconsistent success (Saby *et al.*, 2003; Ye *et al.*, 2008; Coma *et al.*, 2013).It is essential to elucidate the impact of operation conditions such as SIR and influent COD concentration on sludge reduction in OSA to ensure reliable performance for the water industry. Changing SIR varies the residence time of sludge in aerobic/anoxic regimes and may have important implications on sludge reduction mechanisms. However, current information in the literature is inadequate to pin-point the optimum SIR value or range for sludge reduction. Meanwhile, influent COD concentration affects biomass growth and substrate consumption (Tchobanoglus *et al.*, 2003; Gómez *et al.*, 2006). The influent COD of different WWTPs treating domestic sewage may vary due to the presence of primary sedimentation units (Tchobanoglus *et al.*, 2003). The impact of influent COD variation on OSA performance remains to be evaluated. Khursheed *et al.* (2015) observed that increasing the ratio of sludge exposed to anaerobic and aerobic conditions (0-8.24 g $MLVSS_{anaerobic}$/g $MLVSS_{aerobic}$) in OSA enhanced sludge reduction (0-39.8%). Saby *et al.* (2003) investigated the impact of sludge retention time (SRT) in the external anoxic reactor of OSA over a range of 11-17 d and observed 23-58% reduction in biosolids production under longer SRTs or smaller SIRs. The SRT of the anoxic reactor in the study of Saby *et al.* (2003) was significantly longer than that of Ye *et al.* (2008) (5.5-11.5 h), but similar sludge reduction has been achieved by both studies. On the other hand, Sun *et al.* (2010) enhanced sludge reduction of OSA by 24% by interchanging sludge more frequently between an SBR and external anaerobic reactor (from once per day to four times per day). Given the inconsistent trends reported in the literature, it is worthwhile to determine the impact of SIR on sludge reduction. Additionally, a systematic investigation under different

influent COD concentrations will help assess the performance and facilitate the implementation of OSA in WWTPs with and without primary sedimentation. This chapter aims to systematically investigate the impact of SIR on OSA performance at different influent strengths, *i.e.,* using domestic sewage before and after primary sedimentation. Volatile solids content and water quality parameters including COD and nutrient concentrations were monitored during continuous operation of the reactors to elucidate the underlying mechanisms of sludge reduction.

5.2 HYPOTHESIS

- Changing the SIR of OSA may affect sludge reduction mechanisms and consequently, sludge reduction.
- Changing the influent COD may impact sludge reduction in OSA.

5.3 MATERIALS AND METHODS

In this chapter, continuous OSA and control systems were operated in parallel Chapter (3.2). The effect of SIR of OSA on sludge reduction was determined using settled and unsettled domestic sewage as the influent (*i.e.,* feed to the SBRs).

5.3.1 Reactor configuration and operation

Details on the configuration and operation of the laboratory-scale OSA (SBR_{OSA} attached to external aerobic/anoxic and anoxic reactors) and control ($SBR_{control}$ attached to single-pass aerobic digester) systems are described in Chapter 3 (Section 3.2).

To determine the impact of SIR on sludge reduction, the SIR (Figure 5.1) between the external anoxic reactor and SBR_{OSA} (q_5) was adjusted to 11, 16.5, and 22% by volume while using settled sewage as the influent (Table 5.1). The best OSA performance (*i.e.,* the highest reduction in sludge yield of SBR_{OSA} relative to $SBR_{control}$) was achieved at 11%, and thus this condition was also evaluated using unsettled sewage as the influent. To confirm the observed trends with unsettled sewage, the interchange of sludge between SBR_{OSA} and the external anoxic reactor was suspended (*i.e.,* there was no SIR), and then resumed at 11% (Table 5.1). The SRT of SBR_{OSA} and $SBR_{control}$ was maintained 10 d. The total SRT of the external reactors of OSA and the control aerobic digester was maintained at 20 d. $FeCl_2$ was not added to the influent.

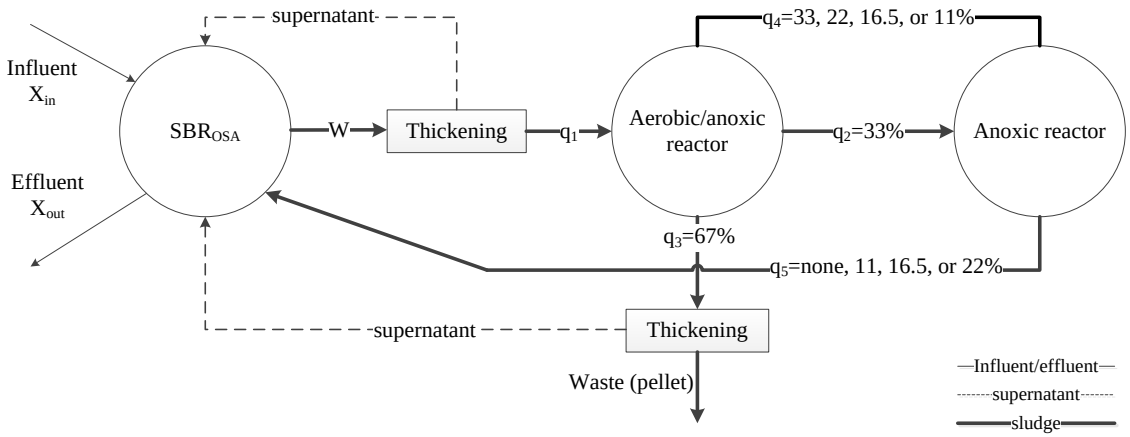

Figure 5.1. Schematic diagram of the OSA system. The SIR between the external anoxic reactor and SBR_{OSA} (q_5) was adjusted to none, 11, 16.5, or 22%. Consequently, the transfer rate of sludge from the anoxic reactor to the aerobic/anoxic reactor (q_4) was 33, 22, 16.5, or 11%, respectively.

Table 5.1. Summary of the experimental phases in this chapter. The SIR (none-22%) and influent (settled and unsettled sewage) were varied while SRT_{SBR} was maintained 10 d, SRT_{ext} was maintained at 20 d, and $FeCl_2$ was not added to the influent.

Experimental phase	Operation period (d)	SIR (%)	Influent [b]
I	43	16.5	Settled sewage
II	78	22	Settled sewage
III	71	11	Settled sewage
IV	52	11	Unsettled sewage
V	39	None [a]	Unsettled sewage
VI	33	11	Unsettled sewage

[a] Sludge interchange between SBR_{OSA} and external reactors was suspended
[b] Influent fed to the SBRs

5.3.2 Domestic sewage

Unsettled and settled sewage were obtained from the Wollongong WWTP fortnightly and stored at 4 °C prior to use. The former was collected at the beginning of the primary sedimentation channel, whereas the latter was collected at the outlet of the same channel. Due to rapid hydrolysis of readily biodegradable solid particles and the higher soluble ammonia concentration

in the unsettled sewage, the average sCOD of the unsettled sewage was significantly higher than that of the settled sewage (Table 5.2).

Table 5.2. Summary of the properties of settled and unsettled sewage. The values are the average ± standard deviation where *n* = number of measurements.

Property	Settled sewage	Unsettled sewage
sCOD (mg/L)	60±32 (*n*=48)	113±87 (*n*=33)
TOC (mg/L)	50.6±21.9 (*n*=48)	49.8±24.2 (*n*=33)
NH_4^+-N (mg/L)	31.2±7.5 (*n*=48)	68.1±31.7 (*n*=33)
PO_4^{-3}-P (mg/L)	26.0±12.0 (*n*=48)	46.7±48.2 (*n*=33)
pH	5.9 (*n*=42)	6.9 (*n*=32)
TSS (g/L)	0.60±0.12 (*n*=48)	0.67±0.08 (*n*=33)
VSS (g/L)	0.17±0.09 (*n*=48)	0.19±0.07 (*n*=33)
VSS/TSS	0.28	0.28

5.3.3 Calculation of sludge reduction

Sludge reduction was calculated as the difference in sludge yield of SBR_{OSA} and $SBR_{control}$. In this chapter, sludge yield *P* is defined as the sludge produced in terms of MLVSS and *C* is the substrate consumed in terms of sCOD. The detailed calculation of sludge yield is described in Chapter 3 (Section 3.4).

5.3.4 Analytical techniques

The solids concentration and SVI of sludge were measured as described in Chapter 3 (Section 3.5.1.1 and 3.5.1.2), respectively. The solids concentration, TOC/TN, sCOD concentration, ammonia concentration, and phosphate concentration of wastewater were measured as described in Chapter 3 (Section 3.5.1.1 to 3.5.1.5). The DO concentration, pH, and ORP of wastewater and sludge were measured as described in Chapter 3 (Section 3.5.1.9).

5.4 RESULTS AND DISCUSSION

5.4.1 Impact of sludge interchange rate

5.4.1.1 Impact of sludge interchange rate on SBR performance

OSA performance was initially investigated using settled sewage that had relatively low "strength" in terms of sCOD (Table 5.2). During this period (Phase I-III), the TOC removal efficiencies of SBR_{OSA} (59.3±34.5%; *n*=48) and $SBR_{control}$ (58.4±31.1%; *n*=48) were almost

identical (Figure A.6). Moreover, the effluent quality of SBR_{OSA} and $SBR_{control}$ were similar to each other in terms of sCOD and ammonia concentration (Figure 5.2). The results indicate that the effluent quality from the main aeration tank (*i.e.*, SBR_{OSA}) was unaffected by any variation in SIR.

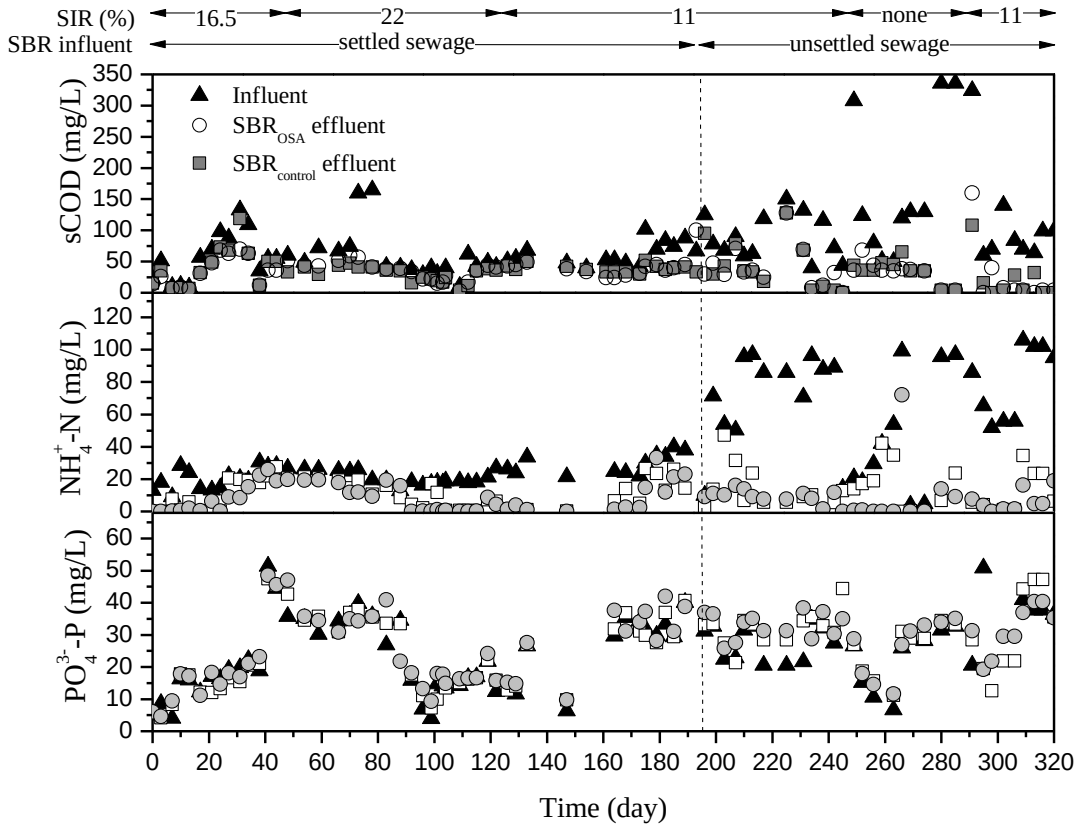

Figure 5.2. sCOD, ammonia, and orthophosphate concentrations in SBR_{OSA} and $SBR_{control}$ at different SIRs (none-22%). The dashed line indicates the change of influent from settled to unsettled sewage.

Neither SBR_{OSA} nor $SBR_{control}$ exhibited orthophosphate removal throughout the operation period (Figure 5.2). The amount of anoxic sludge (orthophosphate concentration=48.4±23.0 mg/L; n=22) interchanged with SBR_{OSA} was relatively low (0.033-0.067 L/day), therefore such interchange did not affect orthophosphate removal by SBR_{OSA}.

The SVI of SBR_{OSA} and $SBR_{control}$ at all SIR was below 100 mL/g (Figure 5.3), confirming that SIR had negligible impact on sludge settleability as reported in a previous study (Semblante *et al.*, 2015).

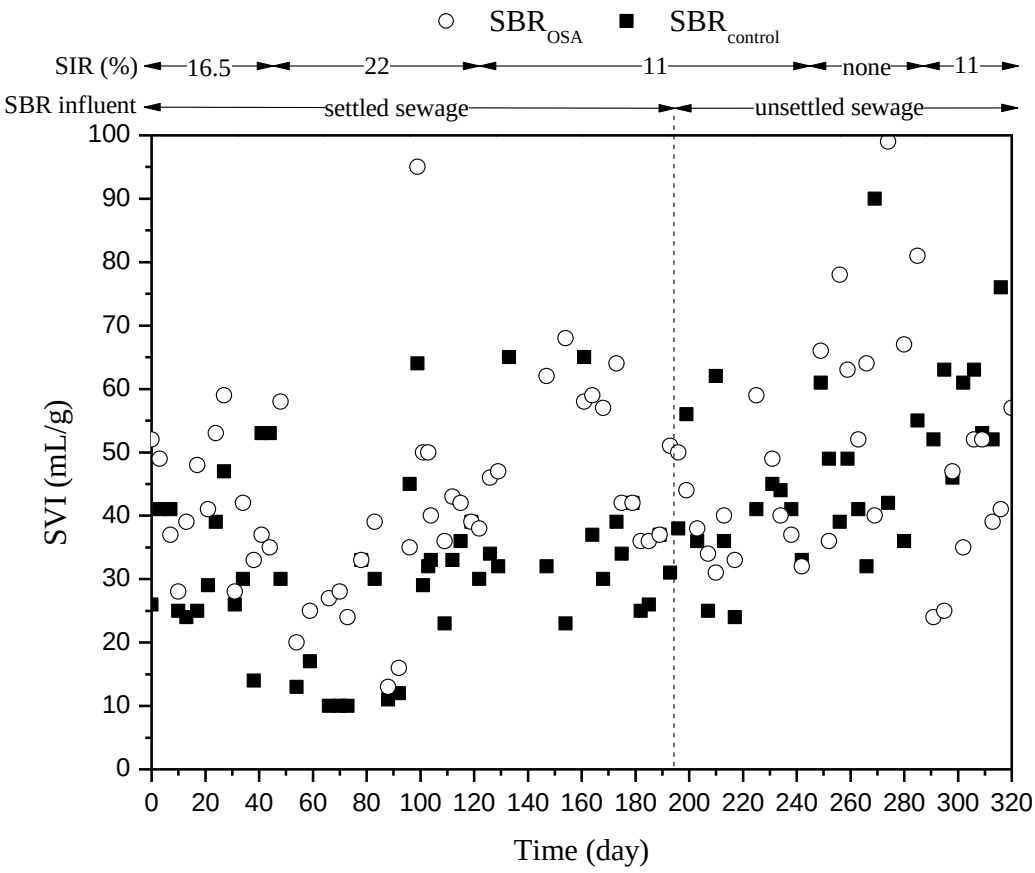

Figure 5.3. SVI of SBR_{OSA} and $SBR_{control}$ at different SIRs (none-22%). The dashed line indicates the change of influent from settled to unsettled sewage.

5.4.1.2 Impact of sludge interchange rate on sludge reduction

A discernible variation in MLVSS of $SBR_{control}$ and SBR_{OSA} occurred due to temporal fluctuations in the sCOD of the settled sewage (Figure 5.4). $SBR_{control}$ maintained a slightly lower MLVSS than that of SBR_{OSA} when SIR was 16.5 and 22%. However, the MLVSS of $SBR_{control}$ became higher than that of SBR_{OSA} when SIR was changed to 11% (Figure 5.4). This is an indication that sludge reduction by $SBR_{OSA}$was enhanced at an SIR of 11%, and the sludge yield data (Table 5.3) further demonstrated this trend.

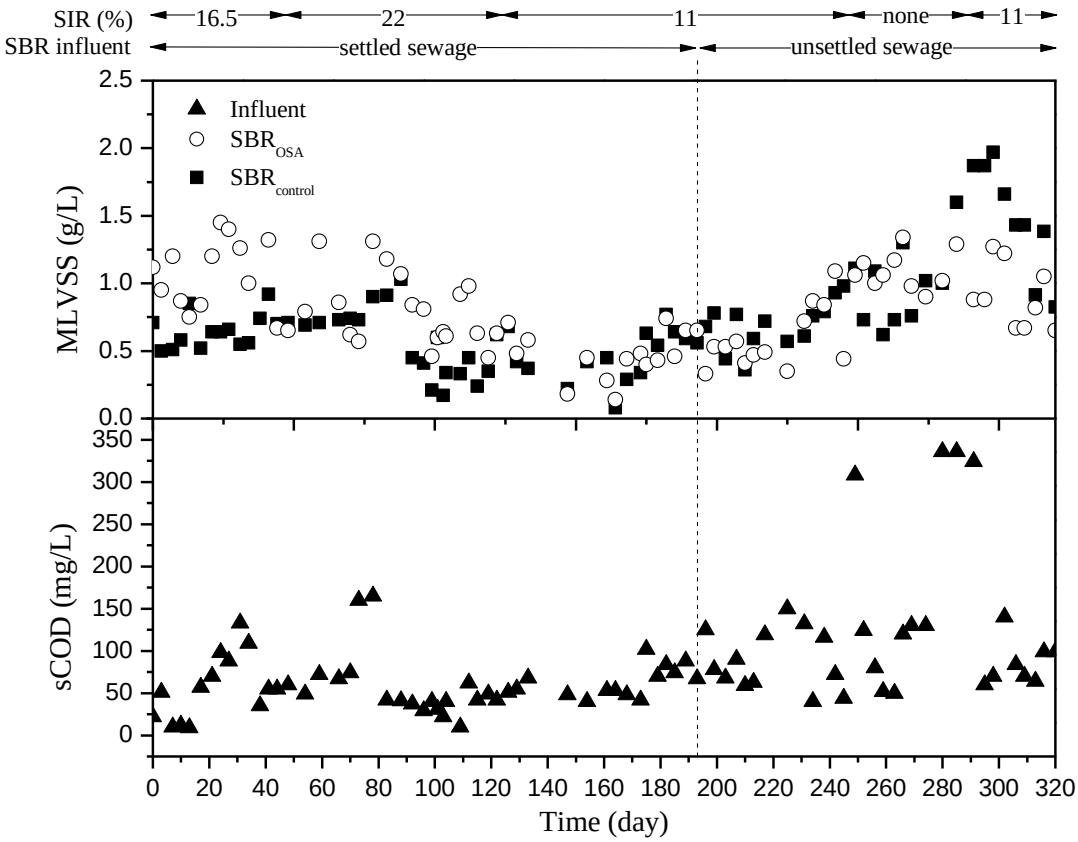

Figure 5.4. Influent sCOD and MLVSS of SBR_{OSA} and $SBR_{control}$ at different SIRs (none-22%). The dashed line indicates the change of influent from settled to unsettled sewage.

Table 5.3. Sludge yield of SBR_{OSA} and $SBR_{control}$ at different SIRs (none-22%) and influent (settled and unsettled sewage). The values are the average ± standard deviation where *n* = number of measurements.

Experimental phase	**Influent**	**Influent sCOD concentration (mg/L)**	**SIR of OSA (%)**	**Sludge yield *Y* (g MLVSS/g sCOD)**				
				$SBR_{control}$	R^2	SBR_{OSA}	R^2	*Reduction (%)*
I	Settled sewage	60±43 (*n*=11)	16.5	10.54	0.85	7.87	0.92	25
II	Settled sewage	58±40 (*n*=20)	22	3.50	0.85	4.01	0.93	None
III	Settled sewage	59±18 (*n*=18)	11	1.54	0.93	0.73	0.78	53
IV	Unsettled sewage	105±68 (*n*=14)	11	0.50	0.60	0.00	0.85	100[b]
V	Unsettled sewage	162±121 (*n*=10)	0	0.14	0.70	0.14	0.78	None
VI	Unsettled sewage	74±36 (*n*=9)[a]	11	1.96	0.93	1.40	0.93	29

[a] sCOD of the unsettled sludge during this run was comparable to that of settled sludge at earlier periods because of wet weather
[b] No excess sludge yield

An SIR of 11, 16.5, and 22 % was equivalent to a residence time of 17.2, 16, and 15 d, respectively, in the external reactors. Comparison of the sludge yield (*Y*) of $SBR_{control}$ and SBR_{OSA} (Figure 5.5) shows that the highest sludge yield reduction (*i.e.*, 53%) was attained at an SIR of 11% (Table 5.3). Notably, there was a large discrepancy in the sludge yield of both SBR_{OSA} and $SBR_{control}$ across Phases I-III. This was probably because sludge production varied with influent characteristics. Indeed, there was significant variation in sCOD at different phases although the average values were comparable. It is also possible that there was difference in wastewater composition (*e.g.*, proteins and polysaccharides) at difference phases. This hypothesis cannot be verified because only sCOD (the bulk concentration of organic compounds) was measured in this part of the study. To avoid over- or underestimation of OSA performance, it is crucial that sludge yields across different studies were not compared with each other. Rather, the sludge yield of SBR_{OSA} and $SBR_{control}$ was compared at each phase only. This approach enables elimination of uncontrollable factors (*e.g.*, diurnal variation in the composition of real wastewater).

Although a systematic investigation of SIR is not available in the literature, the findings of the current study agree with the general trend reported in a few available studies. Saby *et al.* (2003) and Coma *et al.* (2013) suggested that longer treatment of sludge under substrate- and oxygen-deficient conditions may lead to higher sludge reduction in OSA. However, they did not investigate the processes that were impacted by residence time. The current study shows that SIR clearly had an impact on biological reactions in the external reactors of OSA (to be discussed in Section 5.4.3).

Sun *et al.* (2010) achieved a 30% enhancement in sludge reduction by increasing the sludge exchange frequency while maintaining an SIR of 10%. The mechanism behind this trend was not elucidated. Nevertheless, it is possible that faster degradation of the returned sCOD occurred in the SBR at higher return rate (Sun *et al.*, 2010). Further investigation of the combined effect of SIR and sludge interchange frequency is recommended, but that is beyond the scope of this chapter.

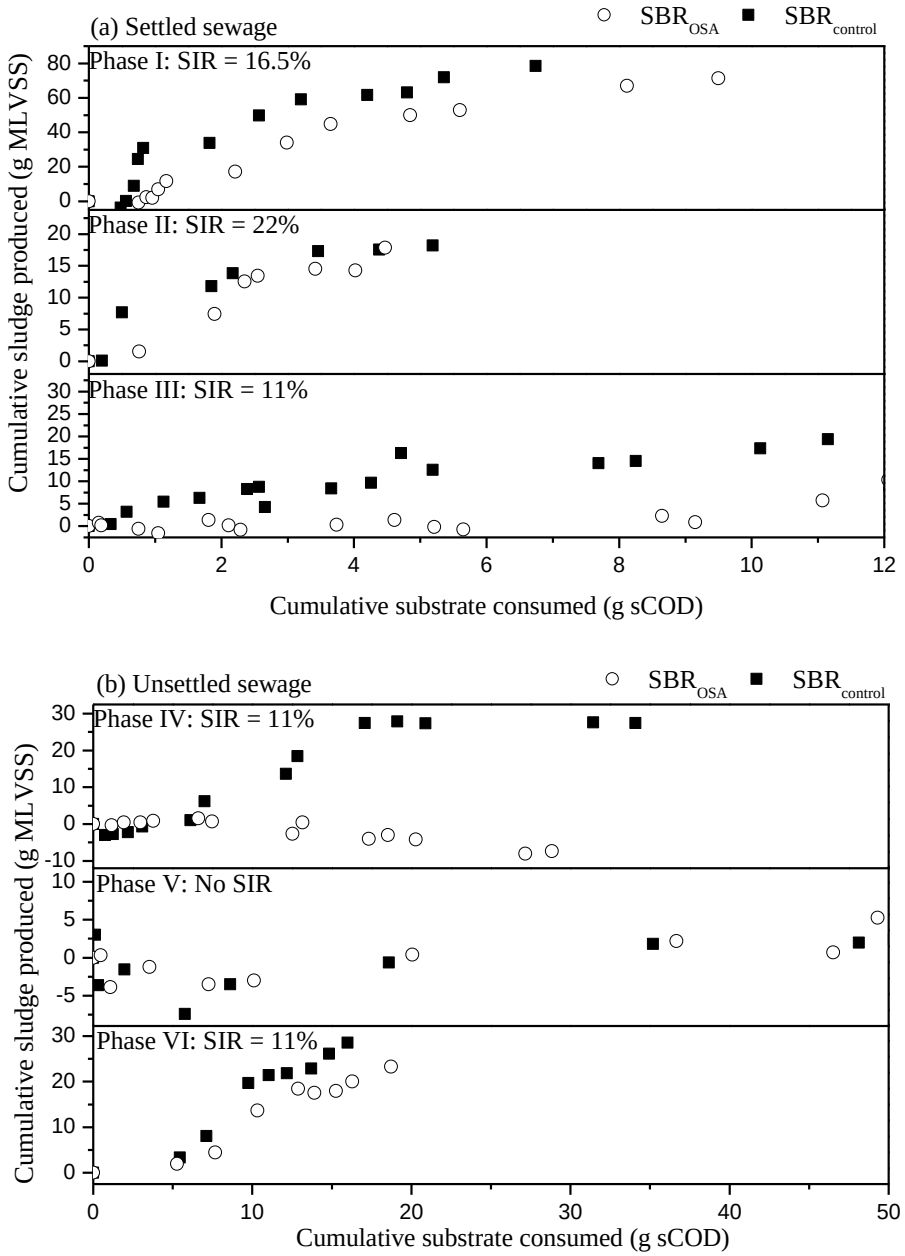

Figure 5.5. Sludge yield of $SBR_{control}$ and SBR_{OSA} at different SIRs (none-22%) when influent was (a) settled and (b) unsettled sewage.

5.4.2 Impact of wastewater strength

5.4.2.1 Impact of wastewater strength on SBR performance

OSA performance was further investigated at higher influent strength by changing the influent provided to the SBRs from settled (sCOD =60±32 mg/L; *n*=48) to unsettled sewage (sCOD =113±32 mg/L; *n*=32) (Table 5.1 and Table 5.2). During this period (Phase IV-VI), OSA was operated with (11%) and without SIR (Table 5.1). Changing the influent did not impact the wastewater treatment performance of the SBRs, *i.e.*, the effluent TOC (Figure

A.6), sCOD, and ammonia (Figure 5.2) concentrations of $SBR_{control}$ and SBR_{OSA} were comparable.

5.4.2.2 Impact of wastewater strength on sludge reduction

With settled sludge as the influent, an SIR of 11% showed the greatest reduction (53%) in sludge yield (Table 5.3). The mechanism behind this is critically discussed in Section 5.4.3. Interestingly, at the same SIR, when unsettled sewage was fed to the SBRs, the sludge yield of SBR_{OSA} decreased further to nearly zero, and thus the calculated sludge reduction was 100% (Table 5.3). This result suggests that OSA is most effective for treatment plants being fed with relatively high strength (*e.g.*, unsettled) sewage. Notably, the sludge yield values of both SBRs were markedly lower when the influent was unsettled sewage than when it was settled sewage. This was because unsettled sewage had a greater fraction particulate COD (pCOD) than settled sewage, and pCOD is potentially biodegradable. In other words, there was relatively high amount of substrate available for biodegradation when the influent was unsettled sewage. To ensure that all biodegradable fraction of wastewater was taken into account, the tCOD of unsettled sewage was determined and used to estimate sludge yield (Table 5.4). A similar pattern was observed when sludge yield was estimated either in terms of sCOD or tCOD: sludge reduction was at the highest level when SIR was 11%.

Table 5.4. Sludge yield of SBR_{OSA} and $SBR_{control}$ as g MLVSS per g tCOD when feed was unsettled sewage. The values are the average ± standard deviation where *n* = number of measurements.

Experimental phase	**Influent**	**Influent tCOD concentration (mg/L)**	**SIR of OSA (%)**	**Sludge yield *Y* (g MLVSS/g tCOD)**				
				$SBR_{control}$	R^2	SBR_{OSA}	R^2	*Sludge reduction (%)*
IV	Unsettled sewage	431±125 (*n*=14)	11	0.52	0.84	0.00	0.86	100
V	Unsettled sewage	437±62 (*n*=10)	0	0.06	0.71	0.06	0.71	0
VI	Unsettled sewage	527±54 (*n*=9)	11	0.18	0.63	0.16	0.59	11

The increase in influent sCOD concentration significantly increased the MLVSS/MLSS ratio of SBR_{OSA} from 0.53±0.10 (n=43) to 0.66±0.11 (n=33) (two sample t-test; $t(65)=4.15$, $p=1.99$, $\alpha=0.05$). There is little information on the effect of MLVSS loading on the external reactors of OSA. A previous study suggested that increasing the MLVSS of sludge treated under aerobic/anoxic conditions enhanced volatile solids reduction (Semblante *et al.*, 2015). Moreover, increasing MLVSS loading reportedly improved the performance of anaerobic digesters possibly by influencing the activity of hydrolysing bacterial groups (Mao *et al.*, 2015).

To determine the performance of OSA at an SIR approaching zero, the interchange of sludge between SBR_{OSA} and external reactors was stopped (*i.e.*, there was no SIR). At this period (Phase V), $SBR_{control}$ and SBR_{OSA} were essentially under the same operation conditions. Therefore, they eventually exhibited a similar sludge yield (Table 5.3). When SIR was resumed at 11% with unsettled sewage (Phase VI), sludge yield reduction by $SBR_{OSA}$was again evident, but it was lower than that achieved in the previous trial (Phase IV). However, this can be attributed to the fact that sCOD concentration of unsettled sewage at Phase VI (*i.e.*, 74±36 mg/L; n=8) was significantly lower compared to that at earlier periods (*i.e.*, 128±96 mg/L; n=24). This pattern reaffirms the recommendation of feeding higher strength sewage to OSA plants.

Unlike the anecdotal use of an SIR of around 10% in the previous studies (Novak *et al.*, 2007; Chon *et al.*, 2011), this current study systematically studied the impact of SIR over a range of 0-22% and showed the greatest sludge yield reduction at an SIR of 11%. The results further demonstrate that the sludge yield reduction can only be ascertained as a range (*e.g.*, between 30 and 100% sludge reduction) depending on wastewater strength, and the beneficial effect of OSA is derived better at higher influent strength as it leads to greater amount of volatile solids undergoing treatment in the external reactors.

Endogenous MLVSS production may occur in the aerobic digester and aerobic/anoxic and anoxic reactors when biomass consumes products of cell lysis (Hao *et al.*, 2010). Therefore, the sludge yield of the OSA (combined SBR_{OSA} and external aerobic/anoxic and anoxic reactors) and control (combined $SBR_{control}$ and aerobic digester) systems were also calculated (Table B.1). A similar pattern was observed, *i.e.*, the greatest sludge reduction occurred at SIR of 11% with either settled or unsettled sewage.

5.4.3 Analysis of sludge reduction mechanisms

To understand the impact of SIR on the sludge reduction mechanism of OSA, the volatile solids, organic, and nutrient concentrations in the aerobic/anoxic and anoxic reactors were analysed. Results show that an intermediate SIR (*e.g.*, 11%) promoted (a) volatile solids destruction in the anoxic reactor (evident in the release of ammonia and phosphate) and (b) nitrification/denitrification in the aerobic/anoxic reactor, ensuring the conversion of lysed biomass into inert products.

5.4.3.1 Observations when influent was settled sewage

The external anoxic reactor was responsible for volatile solids destruction in OSA. The MLVSS/MLSS ratio of the external anoxic reactor (0.45±0.13; *n*=37) was generally lower than that of the aerobic/anoxic reactor (0.51±0.10, *n*=37) and SBR_{OSA} (0.52±0.10, *n*=37) when settled sewage was used as the influent (Figure 5.6). Saby *et al.* (2003) noted that cell lysis in OSA was greater when the external reactor was anoxic (ORP < -150 mV) than when it was aerobic (ORP = 100 mV). Here, greater cell lysis occurred at lower SIR as evidenced by the level of orthophosphate, a product of cell lysis (Goel and Noguera, 2006). Orthophosphate concentration in the external anoxic reactor was 1.75 times higher at the SIR of 11% (52.9±21.5 mg/L; *n*=16) than at 22% (30.1±2.2 mg/L; *n*=4) (Figure 5.7). Results suggest that orthophosphate accumulated (Figure 5.7) in the supernatant possibly because EBPR did not occur under substrate-deficient conditions regardless of SIR. Notably, the release of organic matter and nutrients due to cell lysis was more evident when the MLVSS/MLSS ratio of SBR_{OSA} was higher (*i.e.*, as a result of feeding unsettled sewage), and therefore discussed in more detail in Section 5.4.3.2.

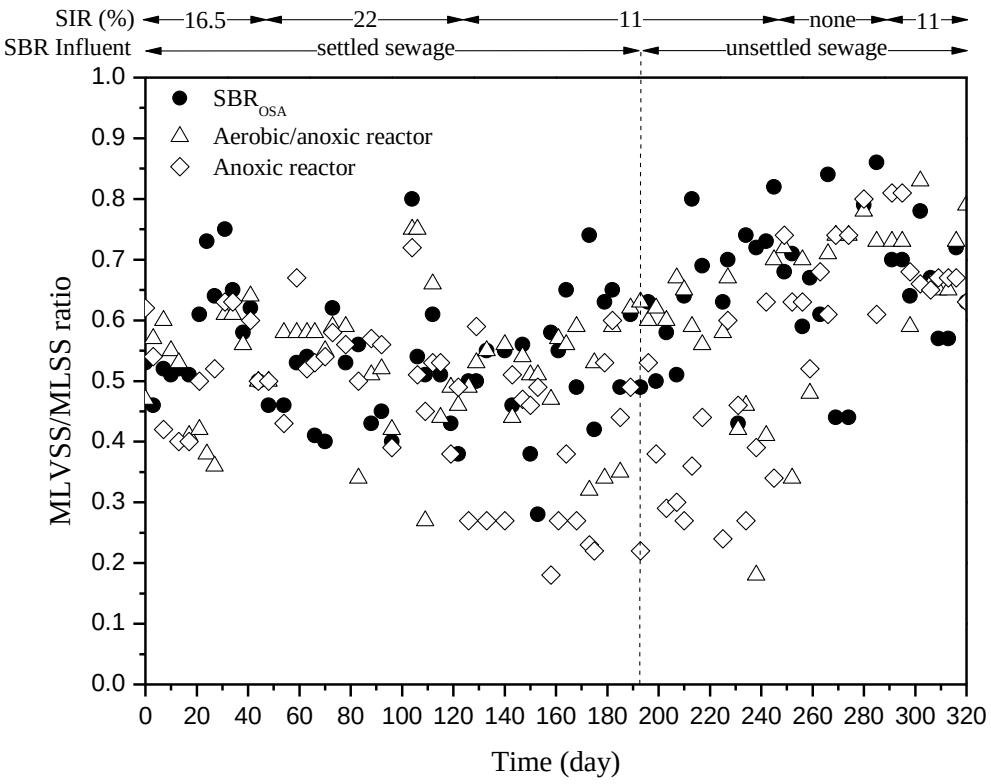

Figure 5.6. MLVSS/MLSS ratio of SBR_{OSA} and external aerobic/anoxic and anoxic reactors different SIRs (none-22%). The dashed line indicates the change of influent from settled to unsettled sewage

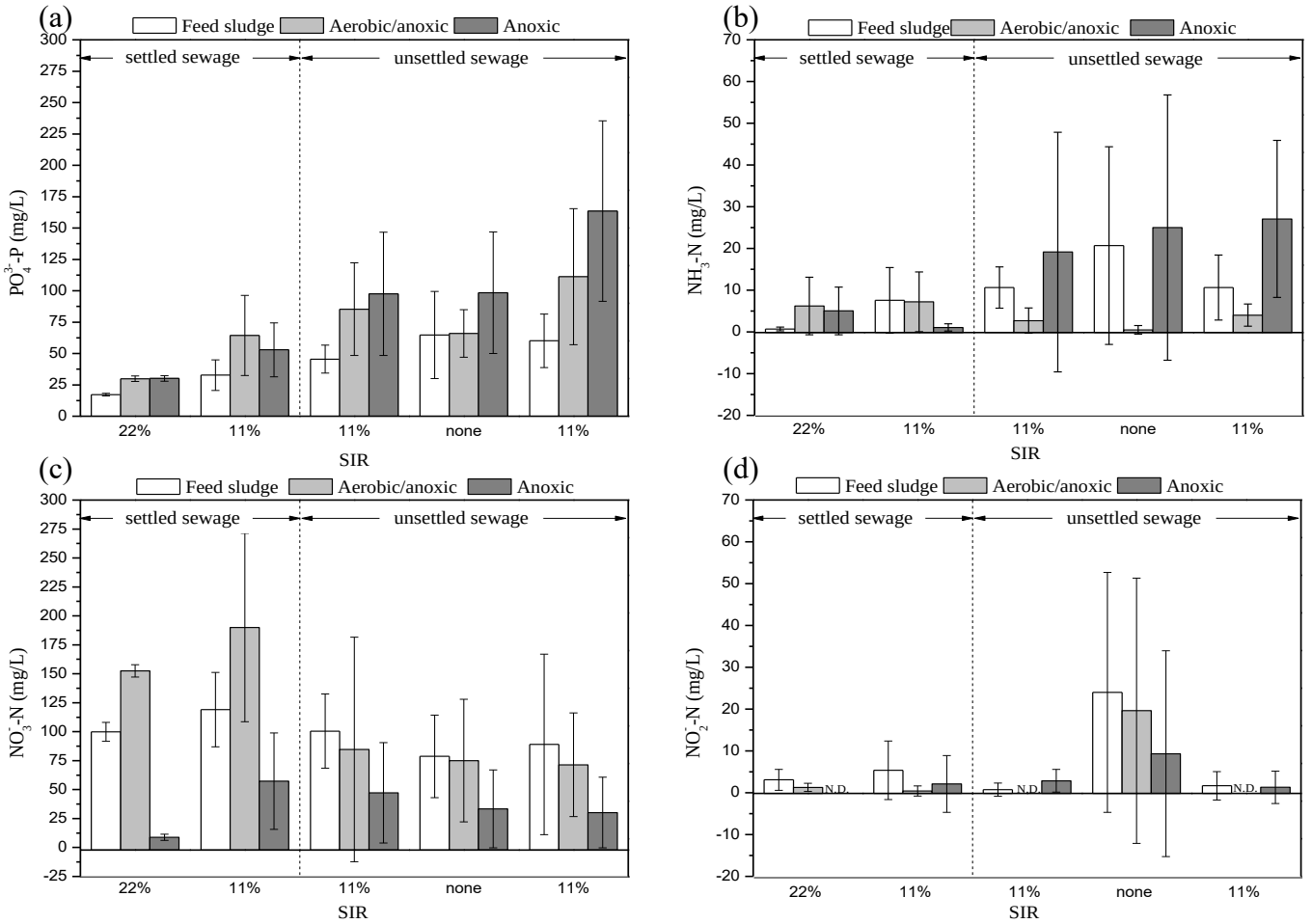

Figure 5.7. Average (a) orthophosphate, (b) ammonia, (c) nitrate, and (d) nitrite concentrations of the supernatants of feed sludge, aerobic/anoxic reactor, and anoxic reactor at different SIRs (none-22%). "Feed sludge" refers to the combined SBROSA and anoxic reactor sludge fed to the aerobic/anoxic reactor. Error bars indicate standard deviation where the number of samples *n*=4 and 17 for SIR of 22% and 11%, respectively (settled sewage); *n*=12, 8 and 9, for SIR of 11, 0, and 11%, respectively (unsettled sewage). The dashed line indicates the change of influent from settled to unsettled sewage.

In addition to cell lysis in the external anoxic reactor, results also reveal the occurrence of nitrification/denitrification in the external aerobic/anoxic reactor. It has been hypothesised that OSA reduces sludge by enriching certain bacteria that are able to lysates (products of cell lysis) (Semblante *et al.*, 2014). These bacteria convert lysates into inert forms (*e.g.*, H_2O, CO_2, and N_2) and consequently decrease organic load during a continuous cycle of sludge interchange (Semblante *et al.*, 2014). This research study found that nitrifying and denitrifying bacteria are among those that are unique enriched in the external reactors of OSA (to be discussed in Chapter 7, Section 7.4.4). A relationship between SIR and nitrification has been observed: a net increase of ammonia occurred in the aerobic/anoxic reactor when SIR was 22% but not when it was 11% (Figure 5.7), indicating that the former condition did not favour nitrification. The low residence time of sludge (*i.e.*, 15 d) in the external aerobic/anoxic reactor at the SIR of 22% could be responsible for the lack of ammonia removal. Nitrification is generally improved by longer sludge residence time. For instance, Li and Wu (2014) found that nitrifiers were enriched in SBRs when SRT was increased from 5-40 d. Likewise, Chuang *et al.* (2015) found that increasing SRT from 10 to 15 d enhanced ammonia removal in an activated sludge-biofilm reactor. More significant removal of ammonia in the external aerobic/anoxic reactor (62-74%) was observed when the influent was unsettled sewage, and this is discussed in greater detail in Section 5.4.3.2.

The SIR of 11% and 22% both resulted to an accumulation of nitrate in the aerobic/anoxic reactor, indicating poor denitrification. This is due to insufficient COD. The theoretical COD/N ratio for biological denitrification is 3.74 (Chiu and Chung, 2003), but the actual COD/N loading ratio into the aerobic/anoxic reactor during this period (*i.e.*, Phases I-III; when the influent was settled sewage) was 1.49-1.70 only. The enhancement of denitrification efficiency at the SIR of 11% was clearly observed when the influent was unsettled sewage (Phases IV-VI; to be discussed in Section 5.4.3.2).

5.4.3.2 Observations when influent was unsettled sewage

Similar to the observation when the influent was settled sewage (Section 5.4.3.1), the average MLVSS/MLSS ratio of the external anoxic reactor was lower than that of the aerobic/anoxic reactor and SBR_{OSA} (Figure 5.6). This low MLVSS/MLSS ratio suggests that the external anoxic reactor is primarily responsible for volatile solids destruction in the OSA system. Furthermore,

the levels of orthophosphate and ammonia in the external anoxic reactor increased by 1.5 times and 7-10 times, respectively (Figure 5.7). Notably, nutrients accumulated in the external anoxic reactor (Figure 5.7), but not sCOD (Figure 5.8). The sCOD that was probably released into the supernatant due to cell lysis was potentially consumed during denitrification (Figure 5.7).

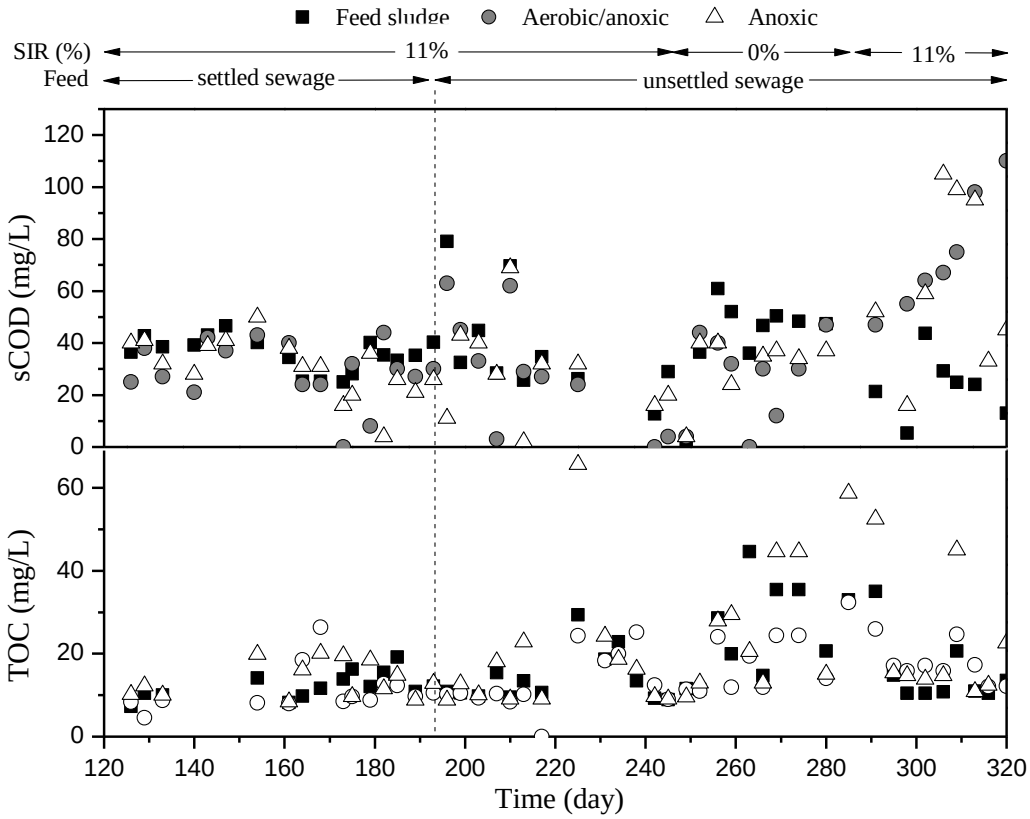

Figure 5.8. sCOD and TOC of the supernatants of feed sludge, aerobic/anoxic reactor, and anoxic reactor at different SIRs (none-22%). "Feed sludge" refers to the combined SBR_{OSA} and anoxic reactor sludge fed to the aerobic/anoxic reactor. The dashed line indicates the change of influent from settled to unsettled sewage.

The highest removals of ammonia (62-74%) and nitrate (17-21%) in the external aerobic/anoxic reactor were observed when SIR was 11% and the influent was unsettled sewage (Phases IV and VI). In contrast, no removal but rather an accumulation of ammonia and nitrate occurred when SIR was 22% (Phase II, discussed in Section 5.4.3.1). This confirms that decreasing SIR increased the residence time of sludge in the external aerobic/anoxic reactor and facilitated nitrification/denitrification (Ye *et al.*, 2008), a processes that converted lysates into inert species (Semblante *et al.*, 2014).

When relatively settled sewage was used as the influent to the SBRs, the denitrification efficiency in the external aerobic/anoxic reactor was negligible at an SIR of 11% probably because of low COD/N loading ratio in the reactor (Section 5.4.3.1). The COD/N loading ratio during the experimental run with unsettled sludge (1.73-2.24) was higher than that of the previous run (1.49-1.70) due to lower nitrate concentration in the feed sludge (Figure 5.7). The increased availability of COD potentially contributed to the enhancement of denitrification in the aerobic/anoxic reactor during this period (Phases IV-VI).

Similar to the observation when the influent was settled sewage (Section 5.4.3.1), biological transformation of orthophosphate was not observed. Instead, orthophosphate accumulated in the external anoxic reactor especially when SIR was 11 % (Figure 5.7), *i.e.*, the condition that favoured cell lysis. This was probably because EBPR did not occur under substrate-deficient conditions. It is also apparent that the release of organic matter and nutrients due to cell lysis was more evident when the MLVSS/MLSS ratio of SBR_{OSA} was higher (*i.e.*, as a result of feeding unsettled sewage). This was possibly due to the increase in the availability of biodegradable material (volatile solids) in sludge.

5.4.3.3 Role of sludge interchange in OSA

In the absence of SIR, there was no influence of the external reactors on the main bioreactor. Consequently, there was no sludge reduction in SBR_{OSA}. On the other hand, suspending the recirculation of sludge between SBR_{OSA} and the external reactors had minimal effect on volatile solids reduction in the external anoxic reactor (Figure 5.6) and nitrification in the aerobic/anoxic reactor (Figure 5.7). However, during this period (Phase V), the concentration of nitrite increased by 5-10 times in the aerobic/anoxic and anoxic reactors (Figure 5.7). Based on the study of Cortez *et al.* (2009), this is a potential indicator of inefficient denitrification. This decreased denitrification in the external aerobic/anoxic reactor was possibly due to insufficient COD in absence of sludge interchange.

The relevance of interchanging sludge in the OSA process is further emphasised when OSA performance is compared with single-pass aerobic or anaerobic digesters. The MLVSS/MLSS ratio of the control aerobic digester (0.55±0.08, n=37 and 0.68±0.08, n=31 when the influent was settled and unsettled sewage, respectively) was slightly higher than that of $SBR_{control}$ (0.52±0.22,

n=37 and 0.69±0.22, *n*=31 when the influent was settled and unsettled sewage, respectively), indicating that aerobic digestion was unable to induce significant volatile solids destruction (Figure 5.9). Furthermore, when sludge circulation between SBR_{OSA} and the external reactors were disconnected (Phase V), the external reactors virtually functioned as single-pass digesters and the sludge yield of SBR_{OSA} became comparable with that of $SBR_{control}$ (Table 5.3). The results of here reinforce previous findings demonstrating that SBRs in OSA systems have lower sludge yield than SBRs attached to single-pass aerobic or anaerobic digesters (Ye *et al.*, 2008; Chon *et al.*, 2011). This suggests that in the absence of sludge interchange between the main bioreactor and external reactors, the mechanism responsible of reducing sludge yield in the main bioreactor is switched off.

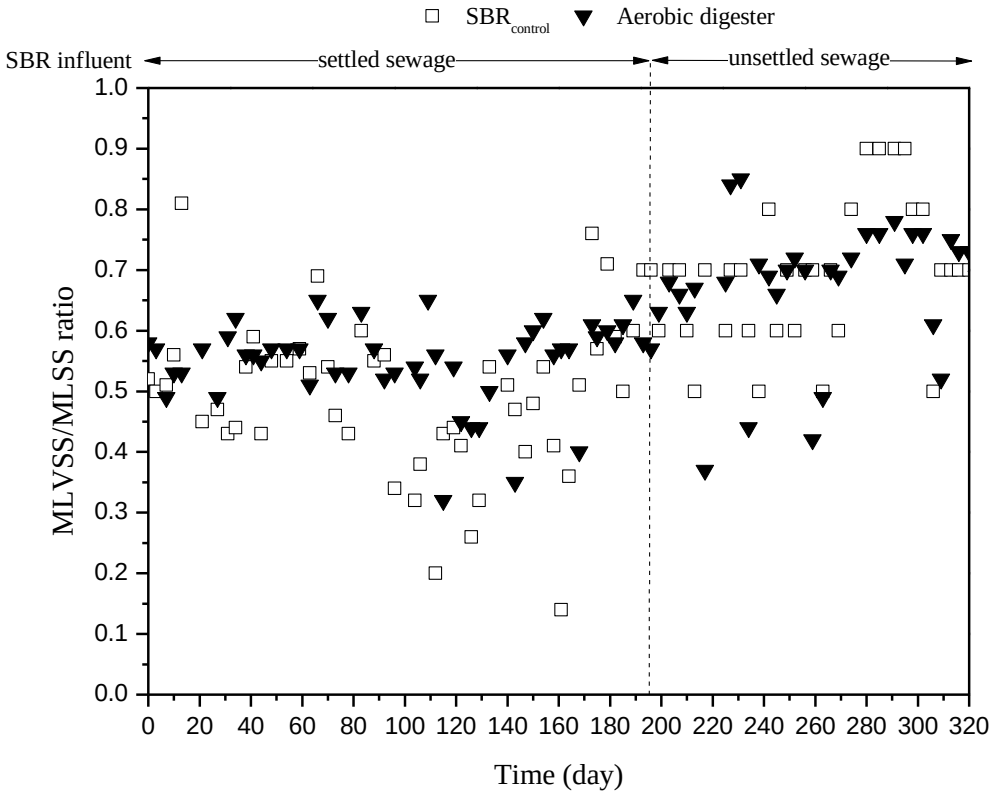

Figure 5.9. MLVSS/MLSS ratio of $SBR_{control}$ and the aerobic digester, which had no sludge interchange throughout the operation period. The dashed line indicates the change of influent from settled to unsettled sewage.

5.5 CONCLUSION

As An intermediate SIR (11%) increased sludge residence time in the external reactors and maximised OSA performance through two mechanisms: (a) providing optimum environment for volatile solids destruction as evidenced by the increase in orthophosphate under anoxic conditions; (b) facilitating the conversion of lysed materials into inert forms as evidenced by the decrease in ammonia and nitrate under aerobic/anoxic conditions. SIRs over 11% showed lower OSA performance, whereas without SIR sludge reduction in the main bioreactor cannot take place. Better OSA performance occurred at higher volatile solids loading to the external reactors. Effluent quality and sludge settleability were unaffected by SIR.

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CHAPTER 6: Effects of sludge retention time (SRT) on sludge reduction in the oxic-settling-anoxic (OSA) process

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6.1 INTRODUCTION

High sludge reduction rates achieved in laboratory-scale OSA operated using synthetic wastewater (Chon et al., 2011a) are rarely observed in pilot- or full-scale systems or when real sewage is used as the feed (Liu, 1996; Liu, 2003). This is probably because of poor operational control stemming from knowledge gaps about the mechanisms governing sludge reduction (Foladori et al., 2010). In Chapter 5, laboratory-scale studies using domestic sewage has demonstrated the key steps in sludge reduction in OSA. Semblante *et al.,* (1992) showed that OSA causes destruction of volatile solids in the external anoxic reactor/s as well as a decline in the sludge yield (i.e., mass of biomass produced per mass of substrate consumed) of the main aeration tank. This finding suggests that sludge reduction may be enhanced by controlling factors that influence sludge autolysis under oxygen- and substrate-deficient conditions in the external reactors of OSA.

Previous research has suggested that sludge reduction by OSA may be mainly due to its long SRT. The addition of external reactor/s that temporarily hold RAS results in an increase of the total SRT of activated sludge (Troiani et al., 2011). SRT is inversely proportional to sludge yield due to the diversion of energy towards cell maintenance rather than synthesis (Quan et al., 2012). However, contradicting reports have been reported regarding the relationship of SRT and OSA performance. For example, Saby *et al.,* (2012) observed that biosolids reduction (23-58%) was directly proportional to the SRT of the external anoxic reactor of OSA (11-17 d). On the contrary, Ye *et al.,* (2003) found that biosolids reduction (14-33%) had an inverse relationship with the SRT of the external anoxic reactor, although the range of SRTs investigated was much shorter (5.5-11.5 h) than that of Saby *et al.,* (2006). These studies were conducted with synthetic wastewater, and furthermore the SRTs reported were scattered, ranging from very short (*e.g.,* less than 1 day) (Ning et al., 2014) to significantly long (*e.g.,* 70-80 d) (Paul et al., 2001). It is difficult to establish a correlation between SRT and OSA performance based on the available literature, especially since the reports are based on varying wastewater, operation conditions, and methods of quantifying sludge reduction.

In addition to reducing biosolids, there is evidence that OSA may affect sludge properties. For example, some studies that used either synthetic (Higgins and Novak, 1997; Tchobanoglus et al.,

2003) or real wastewater report that OSA decreased SVI and improved sludge settleability (Niu et al., 2013). The impact of OSA on sludge dewaterability has not been reported in literature. Sludge dewatering is one of the most challenging downstream processes associated with biosolids treatment. Sludge autolysis in the external reactors of OSA may have implications on sludge dewatering characteristics, but this is yet to be studied systematically.

This chapter aims to determine the impact of SRT of the external anoxic reactors (SRT_{ext}; defined in Chapter 3, Section 3.2.3) on biosolids reduction in an OSA system fed with real wastewater. Volatile solids reduction and associated other biological reactions, namely, release and fate of nutrients in the external reactors were closely monitored. Additionally, this study compares the dewaterability of WAS with and without OSA. A systematic investigation concentrating on these topics has not been reported in literature. The results of this study will shed light on the underlying mechanisms in OSA, and will provide critical information on how OSA performance can be improved.

6.2 HYPOTHESIS

- Changing the SRT_{ext} of OSA may affect sludge reduction.
- Dewatering properties of the final sludge residue produced by the OSA system may be different from that of the control system.

6.3 MATERIALS AND METHODS

In this study, continuous OSA and control systems were operated in parallel as described in Chapter 3 (Section 3.2). The effect of SRT_{ext} on sludge reduction was determined using unsettled domestic sewage as the influent.

6.3.1 Reactor configuration and operation

Details on the configuration and operation of the laboratory-scale OSA (SBR_{OSA} attached to external aerobic/anoxic and anoxic reactors) and control ($SBR_{control}$ attached to single-pass aerobic digester) systems are described in Chapter 3 (Section 3.2).

The SRT_{ext} of both systems was varied (Table 6.1) to determine its impact on sludge reduction. In the OSA system, this was performed by adjusting volume of sludge discarded from the aerobic/anoxic reactor (q_3) (Chapter 3, Section 3.2.1). In the control system, this was performed by adjusting the volume of sludge discarded from the aerobic digester (Q_{out}) (Chapter 3, Section 3.2.2). The SRT of SBR_{OSA} and $SBR_{control}$ (SRT_{SBR}; defined in Chapter 3, Section 3.2.3) was maintained at 10 d. The SIR of the OSA system was maintained at 11%. $FeCl_2$ was not added to the influent.

Table 6.1. Summary of the experimental phases of this chapter. The SRT_{ext} was varied (10-40 d) while the SRT_{SBR} was maintained at 10 d, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage).

Experimental phase	Operation period (d)	SRT_{SBR} (day)	SRT_{ext} (day)
I	52	10	20
II	82	10	40
III	80	10	20
IV	38	10	10

The average conditions in the reactors throughout the operation period are summarised in Table 6.2. The pH, DO, and ORP of the all reactors remained similar throughout the operation period. However, the ORP of the external aerobic/anoxic reactor varied at different SRT_{ext}. The extent and implications of this variation on sludge reduction are discussed in Section 6.4.3.

Table 6.2. Summary of the operating conditions of the reactors in this chapter. The values are the average ± standard deviation where *n* = number of measurements.

Reactor	pH	DO (mg/L)	ORP (mV)
SBR_{OSA}	6.8±0.8 (n=61)	5.4±1.7 mg/L (n=61)	129.7±28.2 (n=34)
Aerobic/anoxic	6.7±0.5 (n=61)	4.6±1.0 / 0.4±0.2 [a] (n=61)	121.6±16.2 / -40.3±17.7 [a,b] (n=34)
Anoxic	6.5±0.3 (n=61)	-	-408±28.6 (n=34)
$SBR_{control}$	6.8±0.6 (n=61)	5.9±2.4 (n=61)	117.7±20.5 (n=34)
Aerobic digester	6.0±1.7 (n=61)	6.2±0.19 (n=61)	202.3±21.5 (n=34)

[a] Measurements were obtained when aeration was turned on/turned off
[b] The ORP of the external aerobic/anoxic reactor varied at different SRT_{ext} (discussed in Section 6.4.3).

6.3.2 Domestic sewage

Domestic unsettled sewage (Table 5.2) was collected from the beginning of the primary sedimentation channel of Wollongong WWTP fortnightly and stored at 4 °C prior to use.

Table 6.3. Summary of the properties of unsettled sewage used in this chapter. The values are the average ± standard deviation where *n* = number of measurements.

Property	Unsettled sewage	*n*
tCOD	474±292 mg/L	61
sCOD	101±54 mg/L	61
TOC	47.2±23.5 mg/L	70
TN	45.0±11.1 mg/L	70
NH_4^+-N	78.4±32.1 mg/L	69
PO_4^{3-}-P	30.3±14.7 mg/L	69
Total P	21.3±14.5 mg/L	28
pH	7.2±0.5	61

6.3.3 Calculation of sludge reduction

Sludge reduction was calculated as the difference in sludge yield of SBR_{OSA} and $SBR_{control}$. In this study, sludge yield *Y* is defined as the cumulative sludge produced in terms of MLVSS (*P*) over the cumulative substrate consumed in terms of tCOD (*C*). It should be noted that in previous chapters, substrate consumption was measured in terms of sCOD because the influent was settled sewage and it had low concentration of particulate matter. In this chapter, the influent was unsettled sewage, which has significant amount of biodegradable particulate matter. To ensure that biodegradable fraction (soluble and particulate) was taken into account, tCOD used to measure substrate consumption. The detailed calculation of sludge yield is described in Chapter 3 (Section 3.4).

6.3.4 Analytical techniques

The solids concentration and SVI of sludge were measured as described in Chapter 3 (Section 3.5.1.1 and 3.5.1.2), respectively. The solids concentration, TOC/TN, sCOD concentration, ammonia concentration, and phosphate concentration of wastewater were measured as described in Chapter 3 (Section 3.5.1.1 to 3.5.1.5). The DO concentration, pH, and ORP of wastewater and sludge were measured as described in Chapter 3 (Section 3.5.1.9). The dewatering properties of sludge (CST and dewatered cake TS) from OSA and control systems were measured as described in Chapter 3 (Section 3.5.2).

6.4 RESULTS AND DISCUSSION

6.4.1 Wastewater treatment performance

The performance of the SBRs was assessed by monitoring influent and effluent tCOD (Figure 6.1) and nutrient concentrations (Figure 6.2). The tCOD concentration of the influent (474±292 mg/L; number of samples *n*=61) had a large variation due to temporal changes in weather patterns (*e.g.*, dilution of wastewater by rainwater). Nonetheless, the tCOD concentration of the effluent of SBR_{OSA} (89±69 mg/L; *n*=61) and $SBR_{control}$ (82±71 mg/L; *n*=61) were similar to each other during the entire operation period. Likewise, SBR_{OSA} and $SBR_{control}$ effluents had similar ammonia and orthophosphate concentrations (Figure 3). SBR_{OSA} and $SBR_{control}$ both exhibited nitrification, removing approximately 90% of influent ammonia. Biological nitrate and orthophosphate removal were not observed in any of the SBRs probably because of the lack of a sufficient anaerobic phase. This shows that OSA would leave the performance of the aeration tank unchanged, which is consistent with previous studies (Chon et al., 2011a; Mishima and Nakajima, 2009) but the current study confirms this over a broader range of influent strength. Nevertheless, this needs to be validated in full scale plant.

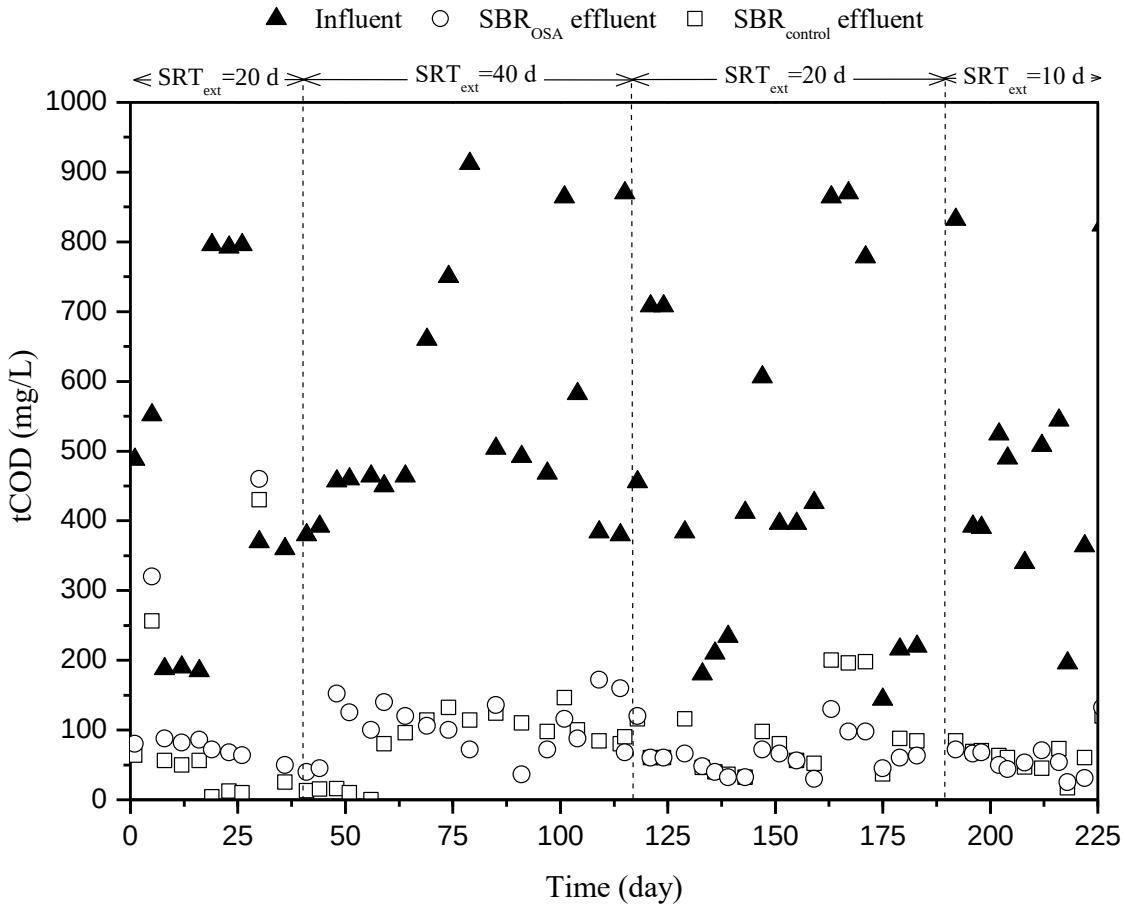

Figure 6.1. tCOD of SBR_{OSA} and $SBR_{control}$ when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d . The dashed lines indicate change in SRT_{ext}.

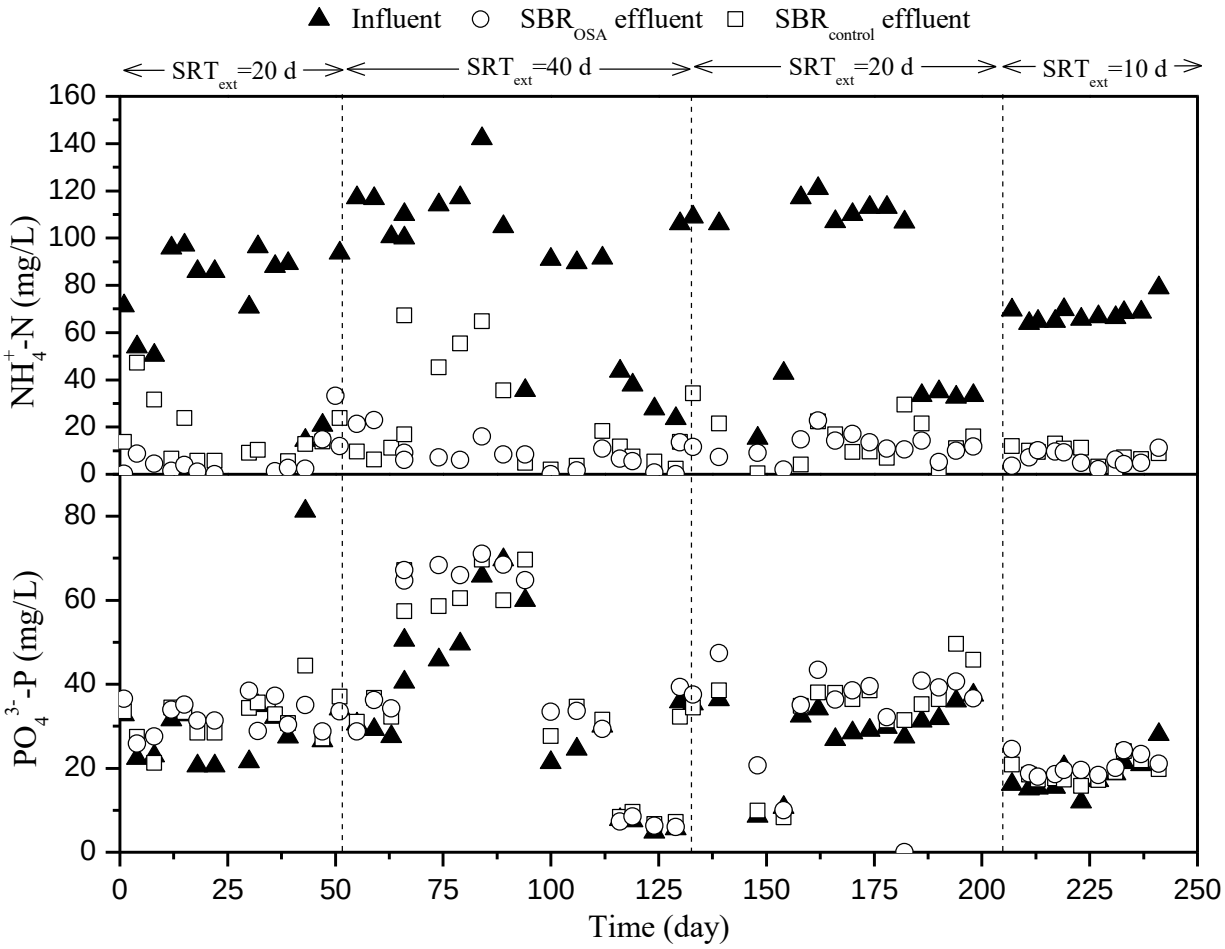

Figure 6.2. Ammonia and orthophosphate concentrations in SBR_{OSA} and $SBR_{control}$ when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The dashed lines indicate change in SRT_{ext}.

6.4.2 Reduction of sludge yield

Results show that increasing SRT_{ext} from 10 to 20 d resulted in sludge yield reduction in SBR_{OSA} from 16 to over 35%. Further increase of the SRT_{ext} to 40 d did not achieve any additional sludge reduction (Table 6.4). In fact the SRT_{ext} of 40 d increased MLVSS concentration in both external aerobic/anoxic (from 1 to 5 g/L) and anoxic (from 0.75 to 3.5 g/L) reactors (Figure 6.3). The sludge yield of the control (combined $SBR_{control}$ and aerobic digester) and OSA (combined SBR_{OSA} and external aerobic/anoxic and anoxic reactors) systems were also compared (

Table B.2), and a similar trend emerged: sludge reduction increased when SRT_{ext} was increased from 10 to 20 d, but did not improve when SRT was further increased to 40 d. These findings suggest the SRT of 40 d results in OSA system failure and thus increases the volatile solids fraction of WAS. In other words, increasing the SRT_{ext} beyond 20 d is counterproductive to sludge reduction in OSA. As discussed further in Section 6.4.3, apparently at an intermediate $SRT_{ext.reactors}$ (20 d) the performance of this particular OSA configuration is maximised.

Table 6.4. Influent tCOD and sludge yield of SBR_{OSA} and $SBR_{control}$ at different SRT_{ext} (*n*=number of samples).

Experimental phase	**SRT_{ext}**	**Influent tCOD concentration (mg/L)**	**Sludge yield *Y* (g MLVSS/g tCOD)**				
			SBR_{OSA}	R^2	$SBR_{control}$	R^2	*Reduction (%)*
I	20	231±125 (*n*=13)	0.00	0.85	0.51	0.84	100
II	40	527±154 (*n*=19)	0.13	0.84	0.13	0.77	0
III	20	478±254 (*n*=12)	0.09	0.69	0.14	0.80	35
IV	10	491±194 (*n*=11)	0.16	0.65	0.19	0.67	16

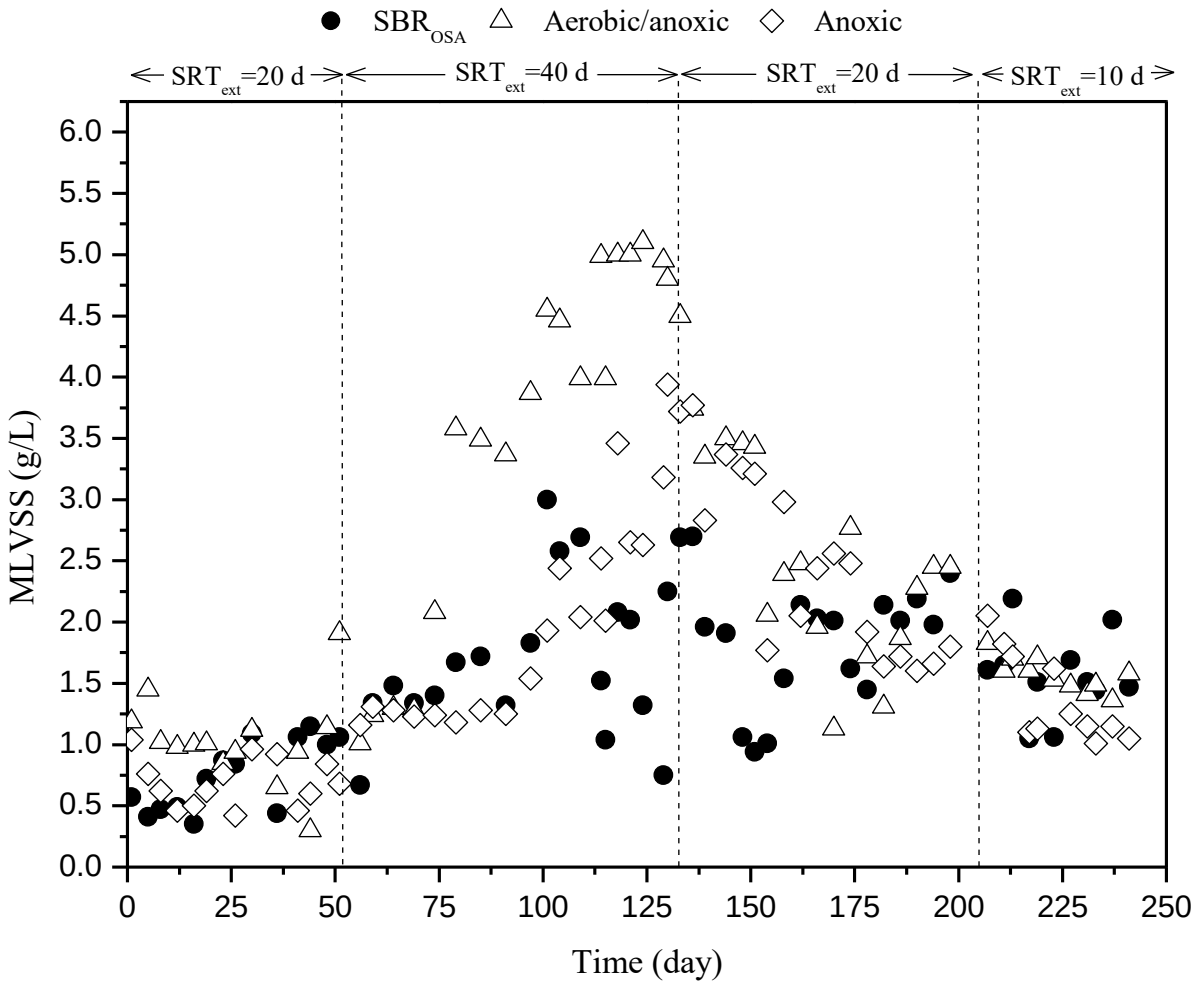

Figure 6.3. MLVSS concentration in the OSA system reactors when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The dashed lines indicate change in SRT_{ext}.

Interestingly, a further advantage of the OSA over the control system was observed upon comparison of their final sludge residue. The MLVSS of WAS of the OSA system discarded from the aerobic/anoxic reactor (Figure 6.3) was up to 65% lower than that of the WAS of the control system discarded from the aerobic digester (Figure 6.4). In other words, sludge produced by OSA is potentially more amenable to stabilization (Novak et al., 2007) and may produce less odour (Yagci et al., 2015) than the sludge produced by the anaerobic digestion.

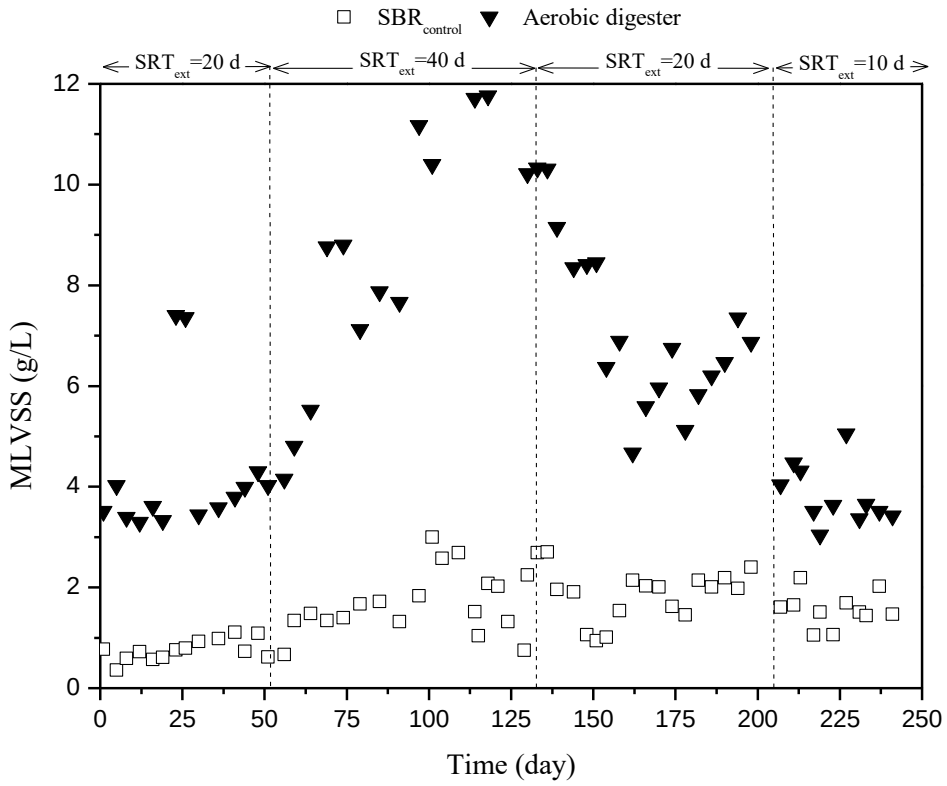

Figure 6.4. MLVSS concentration in the control system reactors when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The dashed lines indicate change in SRT_{ext}.

The optimum SRT_{ext} revealed in this study (20 d) agrees with those found in literature (*e.g.*, 17.4 d reported by Saby *et al.*, (2003)). However, unlike the study of Saby *et al.* (2003) that simultaneously changed the SRTs of the main (5.6-8.7 d) and external reactors (11-17.4 d) at relatively small increments, this study focused on the effect of SRT_{ext} on OSA performance and featured a wider range of experimental conditions (SRT_{ext} =10-40 d) that ensured a systematic investigation. Furthermore, the range of SRTs investigated in this study was significantly broader than those previously reported. For instance, Coma *et al.,* (2007) operated a pilot-scale anaerobic/anoxic/aerobic reactor (SRT=23.5 d) attached to an external anoxic reactor (SRT=0.2-2.3 h), and observed the greatest sludge reduction when SRT_{ext} was 0.2 h. Ye *et al.,* (2015) operated a laboratory-scale SBR (SRT not reported) attached to an external anoxic reactor (SRT=5.5-11.5 h), and observed that an intermediate SRT of 7.5 h minimised the sludge production rate. Both Coma *et al.,* (2013) and Ye *et al.,* (2009) reported that the best OSA performance occurred when SRT_{ext} was kept low (in the range of a few hours), but did not offer

an explanation for their observation. A direct comparison of this study and previous studies is not possible due to variation in operation conditions and system configurations. Nonetheless, this study clearly demonstrates that decreasing SRT_{ext} to 10 d did not favour sludge reduction. The impact of SRT on the mechanism of OSA is discussed in detail in Section 6.4.3.

6.4.3 Mechanism of sludge reduction

It was demonstrated in Chapter 5 (Section 5.4.3) that sludge reduction in this particular OSA configuration (Figure 3.1a) is due to the destruction of volatile solids in the external anoxic reactor, followed by the conversion of destroyed solids into inert products via nitrification/denitrification in the external aerobic/anoxic reactor. In this study, the nutrient concentration (Figure 6.5) and ORP (Figure 6.6) of the external reactors were monitored to provide insight into the effect of SRT on the aforementioned biochemical reactions.

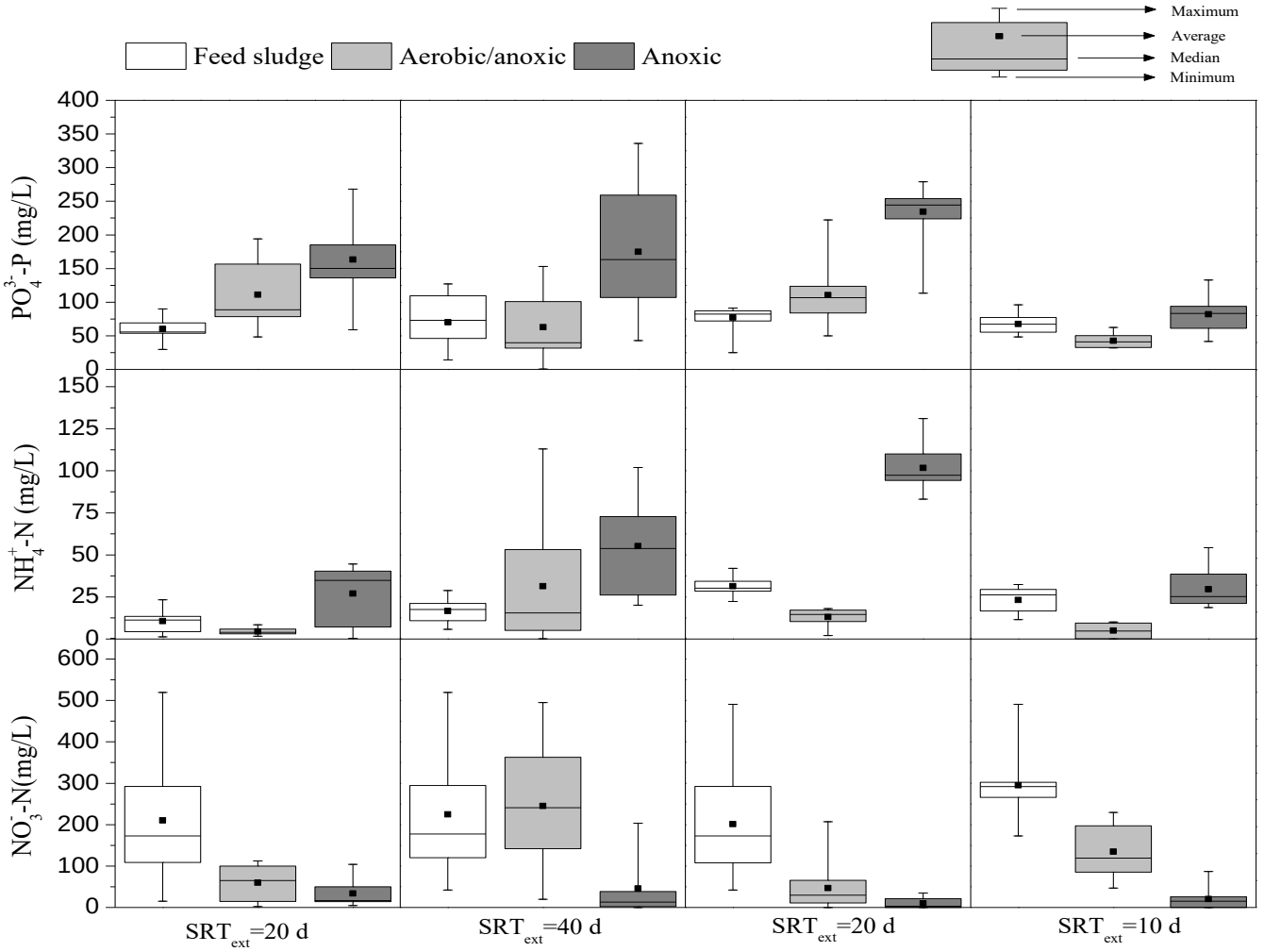

Figure 6.5. Ammonia, orthophosphate, and nitrate concentration of the supernatants of the feed sludge, aerobic/anoxic reactor, and anoxic reactor at different SRT_{ext}. "Feed sludge" refers to the combined SBR_{OSA} and anoxic reactor sludge fed to the aerobic/anoxic reactor. The box plot represents the average, median, maximum and minimum values when SRT_{ext} was varied in the following sequence: 20 (number of samples *n*=13), 40 (18), 20 (16), and 10 (11) d.

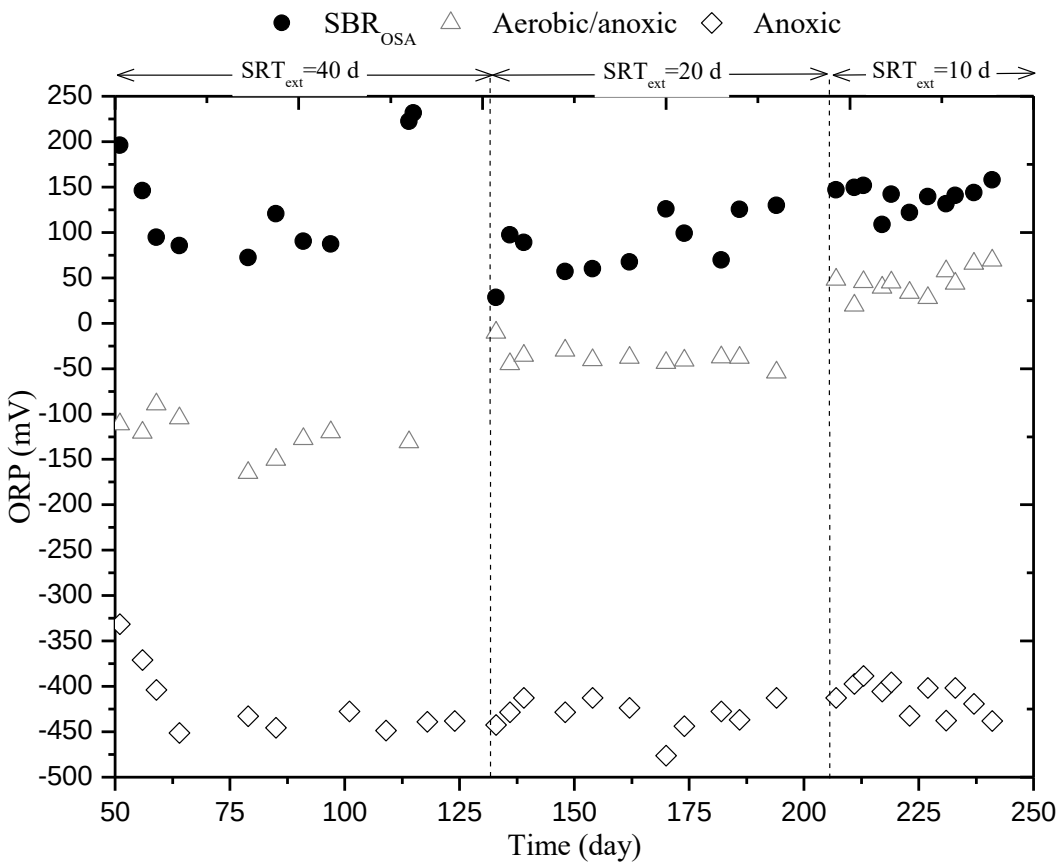

Figure 6.6. ORP of the reactors in the OSA system reactors when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The dashed lines indicate change in SRT_{ext}.

The fact that increasing SRT_{ext} from 10 to 20 d increased volatile solids destruction in the external anoxic reactor but further increasing it to 40 d did not cause any improvement is evident in the release of orthophosphate and ammonia into the mixed liquor supernatant (Figure 6.5). Orthophosphate and ammonia concentrations in the sludge supernatant of $SBR_{control}$ (q_1) and anoxic reactor (q_4) fed into the aerobic/anoxic reactor (Chapter 3, Section 3.2.1) were determined. The ratios of the average concentrations of orthophosphate and ammonia in the external anoxic reactor over that of feed sludge doubled when SRT_{ext} was increased from 10 to 20 d (Table 6.5). The increase in volatile solids destruction can only be due to the enhancement of cell lysis and organic matter biodegradation. However, the ratios were comparable when SRT_{ext} was 20 and 40 d (Table 6.5). This suggests that the maximum autolysis of sludge under oxygen- and substrate-deficient conditions in the external anoxic reactor occurred at the SRT_{ext}

of 20 d. Beyond this SRT, further degradation of the biodegradable fraction cannot take place due to limited availability of electron donors. This is supported by the fact that the ORP of the external anoxic reactor was stable at around -400 mV (Figure 6.6), which suggests that oxidizing agents were always rapidly consumed regardless of SRT.

Table 6.5. The ratios of orthophosphate and ammonia concentration in the feed and external anoxic reactor at different SRT_{ext}.

SRT_{ext} (d)	10	20	40
$PO_4^{3-}{}_{anoxic}/PO_4^{3-}{}_{feed\ sludge}$	1.3	2.7-3.0	2.5
$NH_4^{+}{}_{anoxic}/ NH_4^{+}{}_{feed\ sludge}$	1.2	2.5-3.2	3.3

Previous studies noted that SRT plays a major role in volatile solids destruction during anaerobic digestion of sludge (Chon et al., 2011b; Saby et al., 2003; Yagci et al., 2015). In those studies, optimum SRT ranges were reported based on the enhanced hydrolysis of particulate matter in sludge, resulting in the reduction of volatile solids (Coma et al., 2013; Khursheed et al., 2015). This optimum SRT varies depending on other factors such as digestion temperature and sludge properties, and is usually determined by an empirical approach (Coma et al., 2013; Liu and Tay, 2001; Sun et al., 2010). Generally, the SRT that maximises hydrolysis in anaerobic digestion is relatively short (10 d or less), and therefore hydrolytic reactors are operated under such conditions (Wei et al., 2003). However, there is very little information in literature about the relationship of SRT_{ext} and volatile solids destruction in OSA, and the current study fills in that crucial gap. Interestingly, an analysis of the microbial composition of the sludge reveal that certain bacterial groups (*e.g.*, hydrolysing, fermenting, predatory, nitrifying, and denitrifying bacteria) are able to survive and occupy an ecological niche at oxygen- and substrate-deficient conditions. The implications of microbial composition on sludge reduction are discussed in detail in Chapter 7 (Section 7.4.4).

Nitrification/denitrification occurred in the external aerobic/anoxic reactor when SRT_{ext} was 10 and 20 d, but neither reaction occured at the SRT_{ext} of 40 d (Figure 6.5). Nitrification efficiency, calculated as the difference in the average ammonia concentrations of the feed sludge and the external aerobic/anoxic reactor (Table 6.6), was higher when SRT_{ext} was 10 d (76%) than when it

was 20 d (60-62%). However, as evidenced by the accumulation of ammonia (up to 120 mg/L) in the external aerobic/anoxic reactor, nitrification did not occur when SRT_{ext} was 40 d (Figure 6.5). Firstly, the population of ammonia-oxidizing bacteria, the microorganisms responsible for converting ammonia to nitrite (Liu and Tay, 2001), might have declined as a result of low substrate availability at long residence time. Secondly, the increase in MLSS concentration (Figure 6.3) at long SRT could have decreased the availability of oxygen that is required for nitrification. The available data strongly supports this explanation. The required oxygen/ammonia-nitrogen (mg/mg) ratio for ammonia removal is 1.71 (Daigger, 2014). Nonetheless, in this study, the oxygen/ammonia-nitrogen ratio at the SRT_{ext} of 40 d was only 0.8. This ratio was 2.2 and 1.5 when SRT_{ext} was 10 and 20 d, respectively, indicating that there was greater availability of oxygen for nitrification under those conditions. These findings further suggest that the addition of an aerobic phase in the external reactors facilitated the conversion of destroyed volatile solids to inert materials in OSA, but an appropriate SRT_{ext} needs to be maintained to materialise that advantage.

Table 6.6. The removal of ammonia and nitrate in the external aerobic/anoxic reactor at different SRT_{ext}.

SRT_{ext} (d)	10	20	40
NH_4^+ removal (%)	76	60-62	None
NO_3^- removal (%)	62	15-37	6
mg O_2/mg NH_4^+-N	2.2	1.5	0.8
mg sCOD/mg NO_3^--N	2.9	3.5-3.9	3.6

Denitrification in the external aerobic/anoxic reactor decreased when SRT_{ext} was increased from 20 to 40 d (Figure 6.5). Denitrification efficiency was calculated as the difference in the average nitrate concentrations of the sludge fed from the SBR_{OSA} to the the external aerobic/anoxic reactor and the sludge within that reactor (Table 6.6). The occurrence of denitrification largely depends on the capacity of the preceding nitrification to produce nitrate, and therefore the efficiencies of the two reactions were related. In Chapter 5 (Section 5.4.3.2), it was shown that the denitrification efficiency in the external aerobic/anoxic reactor declined due to insufficient biodegradable COD. In this study, the sCOD/nitrate-nitrogen ratio at different SRT_{ext} were similar to each other (Table 6.6) and were consistently close to the theoretical value of 3.7

(Semblante et al., 2014), which suggests that sCOD would have been available for denitrification throughout the operation period. Therefore, the decline in denitrification at high SRT_{ext} was not due to substrate deficiency. Rather, it is more closely associated with failure of the preceding nitrification reaction in the same tank. An analysis of the microbial composition of sludge confirms that the population of denitrifying bacteria was at the maximum at SRT_{ext} of 20 d (to be discussed in Chapter 7). Denitrifying bacteria may decline at extremely high SRT (Foladori et al., 2010). Another possible cause in the decline of denitrification is the MLVSS in the external aerobic/anoxic reactor (Figure 6.3) that may have hindered the mass transfer of electron acceptor and carbon sources in sludge (Chon et al., 2011a).

As previously observed (Section 5.4.3), orthophosphate accumulated in the supernatant especially in the anoxic reactor (Figure 6.5) where sludge autolysis primarily occurred. This suggests that EBPR did not occur in the anoxic reactor at any SRT_{ext}. Indeed, the dominant bacteria associated with EBPR were not identified when the microbial community structure of sludge at different SRT_{ext} was analysed (Sections 7.4.2).

ORP is a key parameter for regulating sludge reduction in OSA when the sludge is interchanged between aerobic and anoxic conditions. Lower ORP has been associated with greater sludge reduction. For instance, Saby *et al.*, (2011a) reported that increasing the SRT of an external anoxic reactor caused its ORP to decrease from +100 to -250 mV, which helped decrease bacterial count measured using 4',6-diamidino-2-phenyl indole (DAPI) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining techniques. However, in this study, the ORP of the external anoxic reactor was maintained at around -400 mV irrespective of the operation SRT (Figure 6.6). The ORP of the external anoxic reactor remained at a low level because nitrification and denitrification was completed, which is corroborated by the fact that there was minimal ammonia and nitrate in the reactor (see Figure 6.5). However, SRT clearly affected the ORP of the external aerobic/anoxic reactor during the anoxic phase (i.e., when aeration was turned off), which increased from approximately -150 to +50 mV when $SRT_{external\ reactors}$ was decreased from 40 to 10 d (Figure 6.6). This indicates that an OSA configuration involving external aerobic phase that results in an intermediate ORP range (-50 mV) can facilitate sludge reduction.

The results of this study demonstrate that in contrast to previous hypothesis in the literature (Novak et al., 2007; Saby et al., 2003), an extended SRT value in the external reactors is not the

key mechanism responsible for sludge reduction in OSA. Increasing SRT_{ext} from 10 to 40 d enhanced volatile solids destruction in the external anoxic reactor as evidenced by the release of degradation products (orthophosphate and ammonia) into the mixed liquor supernatant. However, an intermediate SRT (20 d) was necessary to convert products of cell lysis into inert products via nitrification/denitrification. Therefore, an intermediate SRT (20 d) maximises the dynamics of the aforementioned reactions. Operation under this relatively short SRT has the additional advantage of minimizing aeration requirements in the external aerobic/anoxic reactor.

6.4.4 Impact of OSA on sludge properties

SBR_{OSA} and $SBR_{control}$ had similar SVI throughout the operation period (Figure 6.7). This indicates that under the operation conditions of this study neither the implementation of OSA nor the manipulation of SRT_{ext} deteriorated the settleability of sludge in the main bioreactor. The dewaterability of sludge was additionally assessed under conditions that facilitated sludge reduction, that is, when SRT_{ext} was 10 and 20 d. Results show that under optimum conditions (SRT_{ext}=20 d), sludge from the OSA system had greater dewatering potential than sludge from the control system. The specific CST of the unconditioned sludge from SBR_{OSA} was lower than that of $SBR_{control}$ (Table 6.7). Likewise, the specific CST of unconditioned WAS_{OSA} was lower than that of unconditioned $WAS_{control}$ (Table 6.7). CST characterises the filterability of slurry-type materials. The rate at which the filtrate is extracted from the slurry is dependent on its resistance, and is inversely proportional to the ease by which moisture can be extracted from the slurry (Saby et al., 2002). The data indicate that it was easier to filter the supernatant from WAS produced by the OSA system than that of the control system.

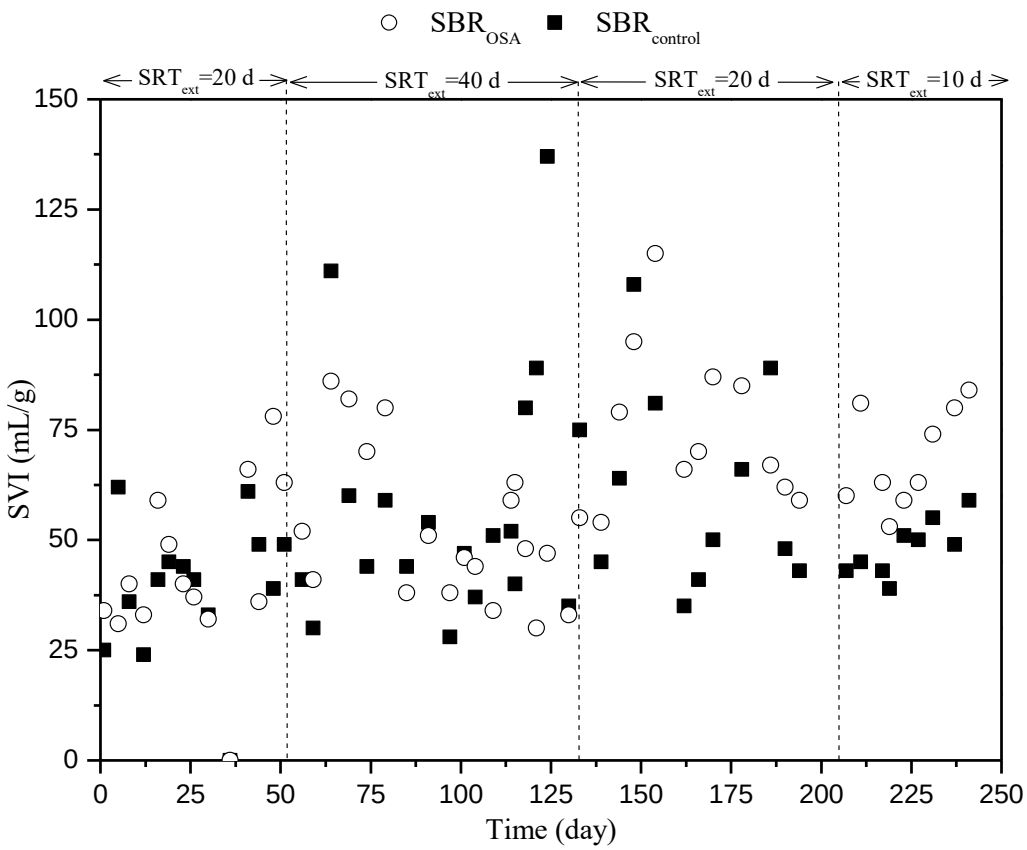

Figure 6.7. SVI of SBR_{OSA} and $SBR_{control}$ when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The dashed lines indicate change in SRT_{ext}.

Table 6.7. Sludge concentration, CST, and TS after dewatering when SRT_{SBR} was 10 d and SRT_{ext} was 10 and 20 d (*n*=number of samples).

SRT_{ext} (d)	Sludge	MLSS (g/L)	MLVSS/ MLSS ratio	CST [c] (sec)	Specific CST [c] (s·L/g MLSS)	Dewatered cake [c] TS (%)
10	SBR_{OSA}	1.47	0.70	7.2±0.3; n=3	4.9	29.2±9.6; n=2
	$SBR_{control}$	2.36	0.75	6.5±0.1; n=3	2.7	20.3±0.4; n=2
	WAS_{OSA} [a]	2.02	0.71	7.7±0.1; n=3	3.8	20.2±1.4
	$WAS_{aerobic\ digester}$ [b]	4.43	0.47	10.6±0.8 ; n=3	2.4	19.8±5.7;n=3
20	SBR_{OSA}	3.24	0.71	10.1±0.2 ; n=3	3.1	17.5±1.7; n=2
	$SBR_{control}$	3.22	0.75	12.3±0.2 ; n=3	3.8	7.2±6.3; n=2
	WAS_{OSA} [a]	3.05	0.71	10.6±0.5 ; n=3	3.5	18.6±3.1; n=4
	$WAS_{aerobic\ digester}$ [b]	6.87	0.74	49.0±1.8 ; n=3	7.1	8.1±5.0; n=4

[a] WAS from the external aerobic/anoxic reactor. This was compared with the sludge from $SBR_{control}$

[b] From the single-pass aerobic digester appended to $SBR_{control}$

[c] CST of unconditioned sludge was measured

[d] TS of dewatered cake was measured. Dewatered cake was produced after conditioning and centrifugation of sludge.

Results also provide evidence that exposing sludge to alternating redox conditions could increase dewatered sludge solids content. Under optimum conditions (*i.e.*, SRT_{ext}=20 d) the dewatered cake TS concentration of WAS_{OSA}, which was the final residue of aerobic/anoxic interchange, was significantly higher than that of $SBR_{control}$, which was solely exposed to aerobic conditions (Table 4). Additionally, the dewatered cake TS concentration of WAS_{OSA} was higher than that of the sludge from the aerobic digester placed after $SBR_{control}$ (Table 6.7). In contrast, when the SRT_{ext} was 10 d, only a marginal difference in the dewatered cake TS concentration of sludge from the OSA and control systems was observed (Table 6.7). Improvement of sludge dewatering by manipulating SRT_{ext} is an important finding of this study because such improvement entail savings in energy and resources for downstream sludge processing and handling.

The disposal and reuse of sludge in Australia is restricted by state regulations. Generally, sludge is classified with (a) contaminant and (b) treatment grade. The heavy metal and organochlorine concentration of WAS_{OSA} was not analysed because these contaminants are not expected in domestic sewage in Australia. Due to aerobic/anoxic treatment, the dewatered WAS_{OSA} has treatment Grade B based on environmental guidelines in New South Wales (EPA-NSW, 2000). If threshold contaminant concentrations were not exceeded, the dewatered WAS_{OSA} is suitable for application in agriculture, forestry, and soil rehabilitation. Alternatively, with this grade, it is acceptable to dispose the dewatered WAS_{OSA} by landfilling (EPA-NSW, 2000).

6.5 CONCLUSION

Under the optimum SRT_{ext}, OSA reduces sludge by facilitating volatile solids destruction in the external anoxic reactor and nitrification/denitrification in the external aerobic/anoxic reactor. Increasing SRT_{ext} facilitated the autolysis of sludge under oxygen- and substrate-deficient conditions. However, beyond the optimum SRT_{ext} (20 d), further biodegradation of sludge did not occur, rather a decrease in nitrification/denitrification efficiency in the external aerobic/anoxic reactor and consequently deteriorated OSA performance was observed. Furthermore, this study showed that aerobic/anoxic sludge interchange helps increase the dewatered cake solids content and reduce the CST of unconditioned sludge when an optimum SRT_{ext} was applied.

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CHAPTER 7: Microbial community structure of the oxic-settling-anoxic (OSA) process and its role in sludge reduction

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7.1 INTRODUCTION

Previous studies have hypothesised that sludge reduction in OSA is driven by the selection of a distinct microbial community brought about by the interchange of sludge between different redox regimes (Chudoba et al., 1992; Coma et al., 2013). PCR-DGGE analysis showed that the microbial community in the anaerobic external reactor of OSA is similar to that of anaerobic digesters (Kim *et al.*, 2012); therefore, reactions such as sulfate reduction and methane production can take place (Saby *et al.*, 2003; An and Chen, 2008). Recently, pyrosequencing analysis showed that OSA systems have greater microbial diversity than control systems (Ye et al., 2008). In particular, Zhou *et al.* (2003) found that fermentative (*Azospira*, *Propionivibrio* and *Sulfuritalea*) and other slow-growing (*Trichococcus* and *Acidovorax*) bacteria were enhanced in the external reactors. Similarly, Ning *et al.* (2003) reported that *Sphingobacteria*, a hydrolyzing order of bacteria, were highly enriched in the external reactors. These studies demonstrate that OSA has a unique microbial diversity (Chudoba et al., 1992; Novak et al., 2007). However, the role of the microorganisms in sludge reduction and the impact of microbial diversity on OSA performance have not been thoroughly explained. Addressing this crucial knowledge gap will be useful in designing bioreactors and selecting operating conditions that are conducive to sludge reduction.

The objective of this study was to determine the microbial community structure in the OSA process to provide insight in its role in sludge reduction mechanisms. To systematically determine the effects of microbial community on sludge reduction, Illumina sequencing analysis was performed when SRT of the SBRs (SRT_{SBR}) was kept constant (10 d) and the SRT of the external reactors (SRT_{ext}; defined in Chapter 3, Section 3.2.3) were varied (10, 20, and 40 d). The potential linkage between operating parameters (*e.g.*, redox condition, SRT_{ext}, and sludge interchange between aerobic and anoxic reactors) and microbial community was determined. Variation in microbial diversity and taxonomic classifications were also systematically investigated.

7.2 HYPOTHESIS

- The microbial community structure of the OSA system may be different from that of the control system.
- The microbial community structure of OSA may play role in sludge reduction.

7.3 MATERIALS AND METHODS

7.3.1 Reactor configuration and operation

Details on the configuration and operation of the laboratory-scale OSA (SBR_{OSA} attached to external aerobic/anoxic and anoxic reactors) and control ($SBR_{control}$ attached to single-pass aerobic digester) systems are described in Chapter 3 (Section 3.2).

The SRT_{ext} of both systems was varied as described in Chapter 6 (Section 6.3.1). In the OSA system, this was performed by adjusting volume of sludge discarded from the aerobic/anoxic reactor (q_3) (Chapter 3, Section 3.2.1). In the control system, this was performed by adjusting the volume of sludge discarded from the aerobic digester (Q_{out}) (Chapter 3, Section 3.2.2). The SRT_{SBR} (*i.e.*, SRT of SBR_{OSA} and $SBR_{control}$) was maintained 10 d. The SRT of SBR_{OSA} and $SBR_{control}$ (SRT_{SBR}; defined in Chapter 3, Section 3.2.3) was maintained at 10 d. The SIR of the OSA system was maintained at 11%. $FeCl_2$ was not added to the influent. A summary of the experimental phases in this chapter is presented in (Table 7.1).

Table 7.1. Summary of the experimental phases in this chapter. The SRT_{ext} was varied (10-40 d) while the SRT_{SBR} was maintained 10 d, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage).

Experimental phase	Operation period (d)	SRT_{SBR} (day)	SRT_{ext} (day)
I	82	10	40
II	80	10	20
III	38	10	10

The average conditions in the OSA and control system reactors at different experimental phases in this chapter are summarised in Table 7.2.

Table 7.2. Summary of the operating conditions of the reactors in this chapter. The values are the average ± standard deviation where *n* = number of measurements.

Experimental phase	SRT_{ext}	Number of samples *n*	Reactor	pH	sCOD [a] (mg/L)	ORP (mV)	DO (mg/L)
I	40	18	SBR_{OSA}	6.5±0.7	–	230±60	6.4±0.6
			Aerobic/anoxic	6.7±0.3	67±49	140±10 / –120±20 [b]	5.5±0.5 / 0.3±0.2 [b]
			Anoxic	6.5±0.4	40±7	–410±20	–
			$SBR_{control}$	6.6±0.4	–	220±20	5.6±0.8
			Aerobic digester	6.3±0.6	109±60	200±70	5.6±1.3
II	20	19	SBR_{OSA}	7.2±0.6	–	230±40	6.4±1.0
			Aerobic/anoxic	6.9±0.4	43±19	90±30 / –40±120 [b]	5.0±1.4/ 0.3±0.1 [b]
			Anoxic	6.5±0.3	45±19	–430±10	–
			$SBR_{control}$	7.3±0.6	–	220±20	6.0±1.2
			Aerobic digester	6.4±0.4	153±23	190±40	6.9±1.1
III	10	11	SBR_{OSA}	6.8±0.4	–	220±40	6.0±0.6
			Aerobic/anoxic	6.2±0.5	60±33	130±60 / 50±20 [b]	3.9±0.2 / 0.3±0.1 [b]
			Anoxic	6.2±0.2	25±60	–390±60	–
			$SBR_{control}$	6.9±0.3	–	220±40	4.9±0.8
			Aerobic digester	6.9±0.5	87±48	120±40	4.7±0.6

[a] Refers to sCOD of the mixed liquor supernatant
[b] ORP and DO measurements when aeration was on/ aeration was off

7.3.2 Domestic sewage

Domestic unsettled sewage with properties described in Chapter 6 (Section 6.3.2) was collected from the beginning of the primary sedimentation channel of Wollongong WWTP fortnightly and stored at 4 °C prior to use.

7.3.3 Calculation of sludge reduction

Sludge reduction was calculated as the difference in sludge yield of SBR_{OSA} and $SBR_{control}$. In this study, sludge yield *Y* is defined as the cumulative sludge produced in terms of MLVSS (*P*) over the cumulative substrate consumed in terms of tCOD (*C*). The detailed calculation of sludge yield is described in Chapter 3 (Section 3.4).

7.3.4 Analytical techniques

7.3.4.1 Wastewater and sludge analysis

The solids concentration of sludge were measured as described in Chapter 3 (Section 3.5.1.1 and 3.5.1.2), respectively. The solids concentration, TOC/TN, sCOD concentration, ammonia concentration, and phosphate concentration of wastewater were measured as described in Chapter 3 (Section 3.5.1.1 to 3.5.1.5). The DO concentration, pH, and ORP of wastewater and sludge were measured as described in Chapter 3 (Section 3.5.1.9).

7.3.4.2 Microbial community analysis

Sludge samples were collected from all the reactors from both the control and OSA systems at the end of Phase I, II, and III of the study (Section 7.3.1). DNA extraction and 16S rRNA gene amplicon sequencing were carried out as described in Chapter 3 (Section 3.5.3.1). Amplicon sequencing was conducted on the Illumina MiSeq platform, utilizing Illumina's Nextera XT Index and Paired End sequencing technology. Sequence analyses were performed as described in Chapter 3 (Section 3.5.3.2).

7.4 RESULTS AND DISCUSSION

7.4.1 Wastewater treatment performance and sludge reduction

The wastewater treatment performance and sludge reduction of the OSA and control systems at different SRT_{ext} are discussed in detail in Sections 6.4.1 and 6.4.2 of Chapter 6, respectively. Briefly, the highest sludge reduction in OSA (35%) was observed at SRT_{ext} of 20 d (Table 7.3). Increasing SRT_{ext} from 10 to 20 d increased sludge autolysis, but further increasing to 40 d did not cause any improvement; rather, it deteriorated nitrification/denitrificatione efficiency in the external aerobic/anoxic reactor indicating that these biological conditions were vital to the conversion of destroyed solids into inert products (Coma et al., 2013). Notably, OSA did not hamper wastewater treatment efficiency of SBR_{OSA} during the operating period (Table 7.3). Although SRText was varied (10-40 d), the effluent quality of SBR_{OSA} was similar to that of $SBR_{control}$ in terms of tCOD, ammonia, and orthophosphate concentration (Table 7.3). These findings strengthen previous assertions that OSA has no effect on wastewater treatment efficiency (Gao et al., 2003; Ye et al., 2008).

Table 7.3. Summary of influent and effluent quality and sludge yield of SBR_{OSA} and $SBR_{control}$ when SRT_{ext} was varied (10–40 d) and SRT_{SBR} was maintained at 10. The values are the average ± standard deviation where *n* = number of measurements.

			Influent and effluent concentration (mg/L)					**Sludge reduction**	
Experimental phase	**SRT_{ext} (d)**	***n***	*Sample*	*tCOD*	*sCOD*	*NH_4^+-N*	*PO_4^{3-}-P*	*SBR_{OSA} sludge yield Y (g MLVSS/g tCOD); (R^2)*	*$SBR_{control}$ sludge yield Y (g MLVSS/g tCOD); (R^2)*	*Sludge yield reduction (%)*
I	40	18	Influent	498±208	105±52	86±36	34±20	0.13; (0.84)	0.13; (0.77)	0
			SBR_{OSA} effluent	78±38	35±19	10±7	40±24			
			$SBR_{control}$ effluent	78±47	43±25	22±22	39±22			
II	20	19	Influent	478±254	99±56	88±38	29±8	0.09; (0.69)	0.14; (0.80)	35
			SBR_{OSA} effluent	75±29	38±13	12±5	33±13			
			$SBR_{control}$ effluent	89±55	44±28	14±11	34±11			
III	10	11	Influent	491±194	132±66	68±4	18±4	0.16; (0.67)	0.19; (0.65)	16
			SBR_{OSA} effluent	59±27	44±22	7±3	21±2			
			$SBR_{control}$ effluent	64±26	47±22	8±4	19±2			

7.4.2 Microbial diversity

7.4.2.1 Comparison of SBR_{OSA} and $SBR_{control}$ microbial diversity

The relationship between microbial community and sludge reduction in OSA was systematically investigated by comparing the diversity indices of SBR_{OSA} (labelled as SBRO) and $SBR_{control}$ (labelled as SBRC) under parallel conditions (*i.e.*, the same experimental phase) (Table 7.4). This approach eliminated potential effects of temporal characteristic variations of real wastewater (Chapter 6, Section 6.3.2) on the microbial communities. Since the SBRs were fed with the same wastewater, the only difference between the two tanks at any particular phase was that SBR_{OSA} interchanged sludge with the external reactors (Chapter 3, 3.2.1) whereas $SBR_{control}$ did not have sludge interchange (Chapter 3, Section 3.2.2).

SBR_{OSA} was more diverse than $SBR_{control}$ when SRT_{ext} was 20 (Phase II) and 40 (Phase I) d (Table 7.4). It was also during these operation periods that the highest diversity indices were recorded for the external anoxic reactor (labelled as ANX) (Table 7.4). This suggests that the microbial makeup of SBR_{OSA} was influenced by the continuous loading of sludge from the external anoxic reactor (described in Chapter 6, Section 6.3.2). In fact, some microbial species were detected exclusively in the OSA system (to be discussed in detail in Section 7.4.4. Nonetheless, high diversity did not necessarily translate to high sludge reduction. For example, SBR_{OSA} had greater diversity than $SBR_{control}$ when SRT_{ext} was 40 d (Phase I; Table 7.4) yet the reactors had similar sludge yield (Table 7.3). A decline in sludge production has been correlated with an increase in microbial diversity in micro-aerobic tanks (Gao et al., 2003), but how they are connected has not been clarified in literature. Current findings suggest that the microbial community structure of SBR_{OSA} shifted to contain more slow-growing bacteria such as nitrifiers (to be discussed in Section 7.4.4.1). These slow growers possibly contribute to the low sludge production rate of SBR_{OSA} relative to $SBR_{control}$. However, the increase in microbial diversity of SBR_{OSA} in itself is not sufficient to explain overall sludge reduction in the OSA system. There is also evidence that cryptic-lysis growth (*i.e.*, sludge autolysis followed by conversion of destroyed solids into inert products) is an important sludge reduction mechanism in OSA (Chapter 5, Section 5.4.3). This is driven by the decay and proliferation of distinct microbial groups in the external reactors. These microbial groups are discussed in more detail in Sections 7.4.4.2 and 7.4.4.3.

Table 7.4. Microbial diversity indices in the OSA and control system reactors. Diversity was estimated at the minimum sequencing depth of all samples (50,000 sequences per sample). Coverage was more than 99% for all samples (data not shown). The values are the average ± standard deviation of 10 iterations (10 random subsampling at sequencing depth of 50,000 sequences per sample.

Experimental phase	SRT_{ext} (d)	Reactor	Sample label	OTUs	Chao1	PD	Shannon
I	40	SBR_{OSA}	SBRO.40	1630 ± 16	2053 ± 72	83.1 ± 0.73	8.2 ± 0.01
		Aerobic/anoxic	AE/ANX.40	1869 ± 12	2330 ± 38	99.9 ± 1.0	7.8± 0.01
		Anoxic	ANX.40	2083 ± 21	2697 ± 68	118.9 ± 1.6	7.9 ± 0.01
		$SBR_{control}$	SBRC.40	1428 ± 25	1832 ± 87	72.1 ± 1.6	7.9 ± 0.01
		Aerobic digester	AE.40	450 ± 7	732 ± 65	34.5 ± 1.0	3.3 ± 0.01
II	20	SBR_{OSA}	SBRO.20	1346 ± 2	1681 ± 10	72.2 ± 0.1	6.40 ± 0.01
		Aerobic/anoxic	AE/ANX.20	1640 ± 9	2078 ± 38	90.6± 0.4	6.6 ± 0.01
		Anoxic	ANX.20	2270 ± 11	2761 ± 42	124.6 ± 0.9	8.5 ± 0.01
		$SBR_{control}$	SBRC.20	1208 ± 9	1527 ± 29	64.0 ± 0.4	7.0 ± 0.01
		Aerobic digester	AE.20	1245 ± 3	1564 ± 16	66.7 ± 0.2	7.3 ± 0.01
III	10	SBR_{OSA}	SBRO.10	983 ± 11	1264 ± 42	54.3 ± 0.5	6.0 ± 0.01
		Aerobic/anoxic	AE/ANX.10	1324 ± 9	1649 ± 40	75.0 ± 1.1	7.3 ± 0.01
		Anoxic	ANX.10	2008 ± 11	2492 ± 39	114.1 ± 1.0	7.9 ± 0.01
		$SBR_{control}$	SBRC.10	1187 ± 7	1450 ± 29	64.2 ± 0.5	6.8 ± 0.0
		Aerobic digester	AE.10	1056 ± 10	1314 ± 37	57.4 ± 1.0	6.7 ± 0.0

7.4.2.2 Microbial diversity of SBR_{OSA} and attached external reactors

To determine the relationship between SRT_{ext} and microbial community in OSA, the microbial diversity indices of the OSA system (SBR_{OSA} and the attached aerobic/anoxic and anoxic reactors) were compared under parallel conditions. The order of increasing diversity was the same at all SRT_{ext}: SBR_{OSA} < intermittent aerobic/anoxic (labelled as AE/ANX) < anoxic. This suggests that diversity was affected by redox condition or ORP level. A decrease in ORP generally indicates the depletion of DO in the mixed liquor (Table 7.2). The diversity of activated sludge (Khanal and Huang, 2003; Saby et al., 2003) and other biological matrices (*e.g.*, marine estuaries) (Chudoba et al., 1992) has been found to intensify when DO concentrations decrease. Microbial diversification at low DO concentration has been primarily attributed to the enrichment of facultative anaerobes (Chudoba et al., 1992) and other microbial groups that can thrive with limited oxygen, though other factors such as appearance of ciliated protozoa (Wang et al., 2008) and bacterial predators (Saby et al., 2003) are potentially relevant as well. In the current study, unique phyla that encompass fermentative, hydrolyzing and predatory bacteria were detected at low DO concentrations (to be discussed in Section 7.4.4).

Bacteria must be exposed to starvation conditions to facilitate autolysis in OSA (Coma et al., 2013; Saby et al., 2003). Indeed the sCOD in the external reactors were 40–50% and 90–95% lower than the sCOD and tCOD of the influent, respectively, implying that readily biodegradable substrate had already been consumed in the main aeration tank. Previous studies have shown that the external reactors of OSA possess a greater variety of microbial species than the main aeration tank (Saby et al., 2003), but the role of diversity in sludge reduction has not been fully elucidated. The results of this study imply that even though a fraction of the biomass undergoes decay under oxygen- and substrate-deficient conditions, microbial groups that are able to utilise lysates (*i.e.*, products of cell lysis) or other food sources are enriched and eventually occupy a niche under environmental stress. These include hydrolyzing, fermentative, denitrifying, and predatory bacteria. The population of these microbial groups, specifically denitrifying and predatory bacteria, changed with SRT_{ext} and led to variation in sludge reduction. This is further discussed in Section 7.4.4.

7.4.2.3 Microbial diversity of $SBR_{control}$ and aerobic digester

Under parallel conditions, the diversity of $SBR_{control}$ was similar to that of the single pass aerobic digester (labelled as AE) when SRT_{ext} was 10 (Phase III) and 20 d (Phase II) (Table 7.4). On the contrary, the attached external reactors that had different redox regimes exhibited greater diversity than aerobic SBR_{OSA}. Notably, the aerobic digester was also under starvation conditions like the external reactors appended to SBR_{OSA}, but it had high DO concentration (>5 mg/L) like $SBR_{control}$, and under the operating conditions of this study, the aerobic digestion did not reduce sludge. This indicates that the deficiency of both readily biodegradable substrate (which occurred in both external reactors of OSA and control aerobic digester) and oxygen (which occurred in external reactors of OSA only) were necessary to shift the microbial community structure and induce sludge reduction. Notably, the sCOD of the aerobic digester was approximately two times higher than that of the external reactors of OSA (Table 7.2) and $SBR_{control}$ effluent (Table 7.3). This could suggest that non-biodegradable organic matter accumulated in the aerobic digester but was not consumed by surviving bacteria.

The diversity of the aerobic digester when SRT_{ext} was 40 d (Phase I) was lower than when it was 10 and 20 d (Table 7.4). Sludge with a long SRT (*e.g.*, >60 d) tended to have high diversity because slow-growing bacteria have more opportunity to propagate (An and Chen, 2008). However, the diversity of aerated systems can also decrease when SRT is increased (*e.g.*, from 2 to 8 d) because the biomass stabilises and microorganisms stop competing for resources (Troiani et al., 2011). In this study, the decline in aerobic digester diversity at SRT_{ext} of 40 d coincided with the proliferation of the order *Xanthomonadales* that accounted for 72% of the biomass (to be discussed in Section 3.4.4). ORP and nutrient levels did not vary significantly in this phase (Table 2), but a slight change in pH possibly caused the proliferation of *Xanthomonadales* and other specific bacteria (to be discussed in Section 7.4.4).

7.4.3 Impact of operational parameters: microbial community and sludge reduction

PCoA is a multidimensional scaling method that shows the similarity or dissimilarity of groups of data using a matrix. In this study, PCoA was utilised to show the clustering of samples based on the differences in unweighted UniFrac distances (Figure 2). Close clustering indicates relative similarity in phylogenetic structure of the samples. Results show that no single operating

parameter can consistently explain the variation of unweighted UniFrac distances between samples. The first two principal components (PC1, PC2) accounted for 43% of sample variation (Figure 7.1). However, a clear clustering of samples corresponded to redox condition, *i.e.*, strictly aerobic (SBR_{OSA}, $SBR_{control}$, and the aerobic digester), intermittent aerobic, and strictly anoxic (Figure 7.1). This indicates that samples with the same redox condition had similar diversity compared to those with same SRT_{ext} or to those that were connected through sludge interchange. This suggests that redox condition had greater influence on diversity compared to SRT_{ext} or sludge interchange.

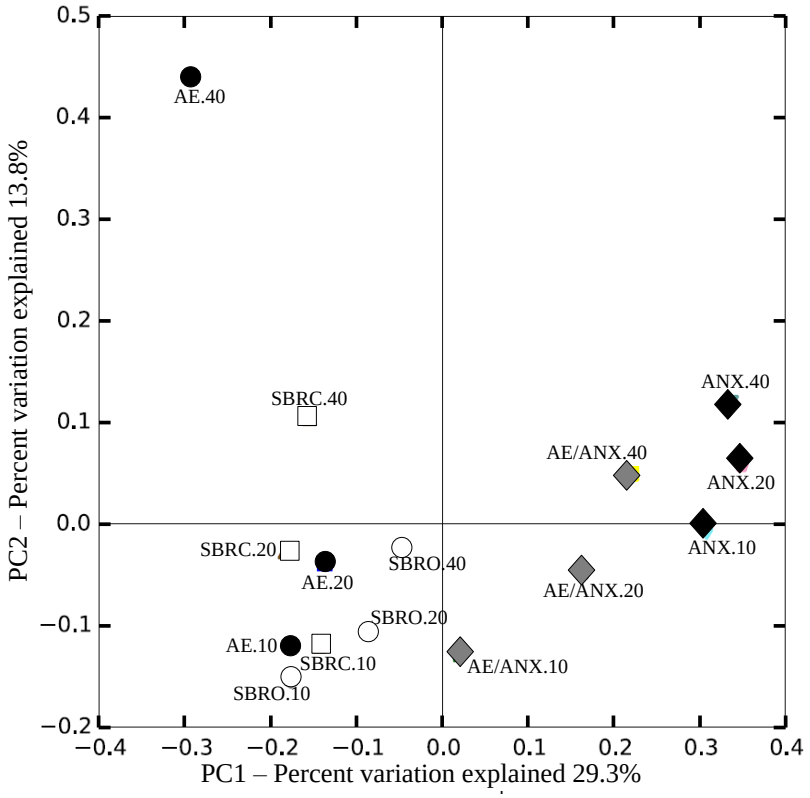

Figure 7.1. Principal coordinates of the unweighted UniFrac calculated at even sequencing depth of 50,000 sequences per sample. The samples were labelled as X.Y, where X = reactor name and Y = SRT_{ext} (d). SBR_{OSA}, aerobic/anoxic reactor, and anoxic reactor of the OSA system were abbreviated as SBRO, AE/ANX and ANX, respectively. $SBR_{control}$ and aerobic digester of the control system were abbreviated as SBRC and AE, respectively.

To further clarify the influence of operation parameters (*i.e.*, redox condition, SRT_{ext}, and sludge interchange between aerobic and anoxic reactors) on the variation of microbial community

structure, constrained analysis of principal coordinates (CAP) and Adonis were applied (Figure 7.2). The constrained model of redox condition, SRT_{ext} and sludge interchange (*i.e.*, OSA system *vs.* control system) showed a significant contribution of redox condition and SRT_{ext} to the first two components in PCoA clustering of samples. For example, constraining redox condition and SRT_{ext} (Figure 7.2) explained nearly 85% as much variation as the first two unconstrained principal components of PCoA (*e.g.*, 29% + 14% in Figure 7.1 *vs.* 27% + 10% in Figure 7.2). Moreover, analysis of variance of unweighted UniFrac distance (Adonis) showed the contributions of redox condition (27%), sludge interchange (16%) and SRT_{ext} (12%) to the difference between microbial communities (Table 7.5). The major role of redox condition on the development of microbial community in the external reactor of OSA systems was also found in previous studies (Chen et al., 2003; Chudoba et al., 1992).

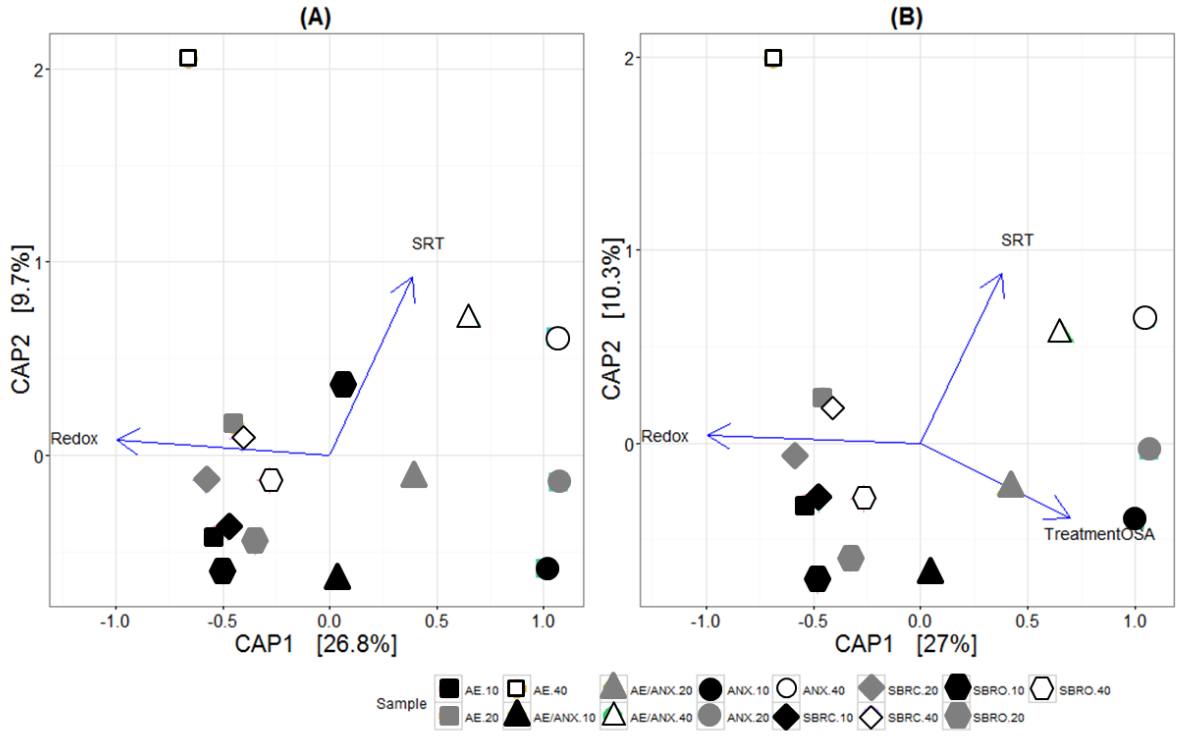

Figure 7.2. Constrained analysis of principal coordinates (CAP) where PCoA-oriented unweighted UniFrac distance was constrained by operating parameters: (A) Redox and sludge retention time (SRT); (B) Redox, SRT and sludge interchange between aerobic and anoxic reactors.

Table 7.5. Adonis (permutational multivariate analysis of variance using distance matrices) of unweighted UniFrac was conducted to find the explanation for the difference between bacterial communities. The analysis was performed by using "vegan" package implemented in R software.

Operational parameters	R^2	p values
ORP	0.27	0.001
Treatment (control *vs.* OSA)	0.16	0.002
SRT	0.12	0.045

Unweighted UniFrac distances (Figure 7.3) showed that at SRT_{ext} of 20 and 40 d, the microbial community structure of SBR_{OSA} and $SBR_{control}$ were more similar to each other than with their respective external reactors (Figure 7.2). This was probably because of interchange of lower volumes of sludge between SBR_{OSA} and the external anoxic reactor at higher SRT_{ext}. Indeed, the microbial community of SBR_{OSA} was closer to that of the external aerobic/anoxic reactor when SRT_{ext} was 10 d.

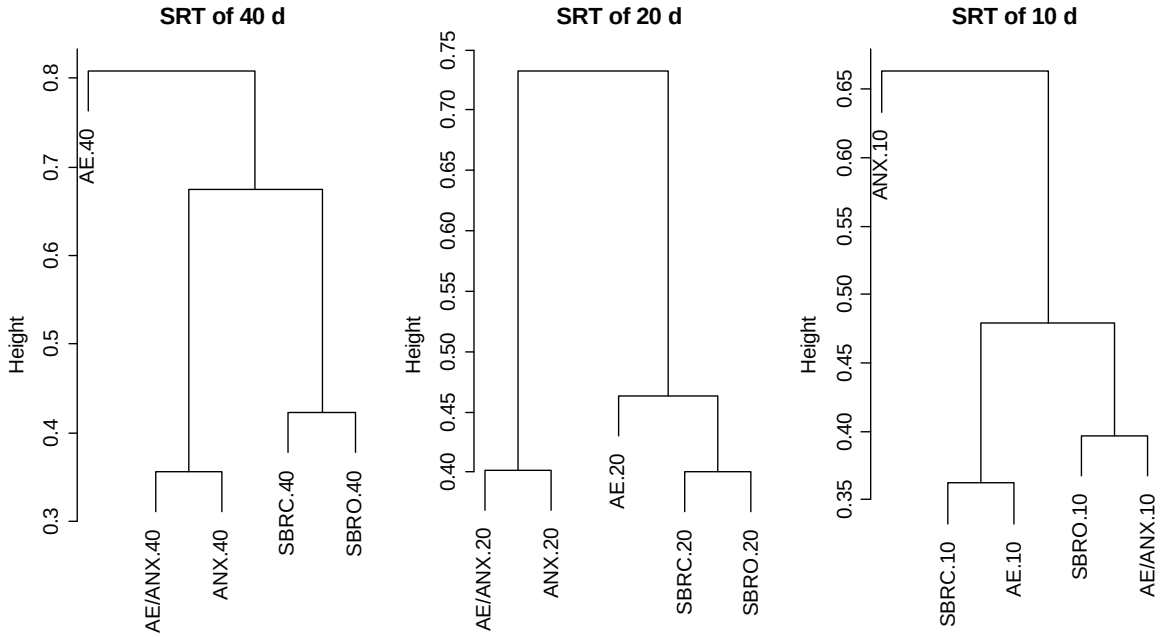

Figure 7.3. Sample clustering based on the unweighted UniFrac distance (calculated at even sequencing depth of 50,000 sequences per sample) at each SRT_{ext} condition. The samples were labelled as X.Y, where X = reactor name and Y = SRT_{ext} (d). SBR_{OSA}, aerobic/anoxic reactor, and anoxic reactor of the OSA system were abbreviated as SBRO, AE/ANX, and ANX, respectively. $SBR_{control}$ and aerobic digester of the control system were abbreviated as SBRC and AE, respectively. The clustering (hclust) method used was "ward.D2."

PCoA and clustering based on unweighted UniFrac showed that each unit of the OSA system sustains the development of a unique microbial community according to redox regimes. SRT_{ext} and sludge interchange between aerobic and anoxic reactors contributed to the dynamics of microbial communities between samples that explained the sludge reduction performance of each unit as well as the systems. The correlation between variation of microbial community and the system performance was clarified further by examining more closely the shift in microbial phyla especially on the important functional groups in Section 7.4.4.

7.4.4 Taxonomic classification and analysis

A total of 43 bacterial phyla and two archaeal phyla with relative abundance of less than 1% were detected in the OSA (SBR_{OSA} and external aerobic/anoxic and anoxic reactors) and control systems ($SBR_{control}$ and aerobic digester) (Figure 7.4). *Proteobacteria* was the most dominant phylum (35–79%) with *γ-, β-*, and *α-Proteobacteria* as the predominant classes (23 ± 11%, 21 ± 10%, and 9 ± 3% (n = 14), respectively). The second most abundant phylum was *Bacteroidetes* (17 ± 10%; n = 14) with *Sphingobaceriia* as the major class (11 ± 8%; n = 14). Our observation is similar to previous reports that *Proteobacteria* and *Bacteroidetes* dominate both CAS and OSA systems involving anoxic external reactors (Wang et al., 2008; Ye et al., 2008).

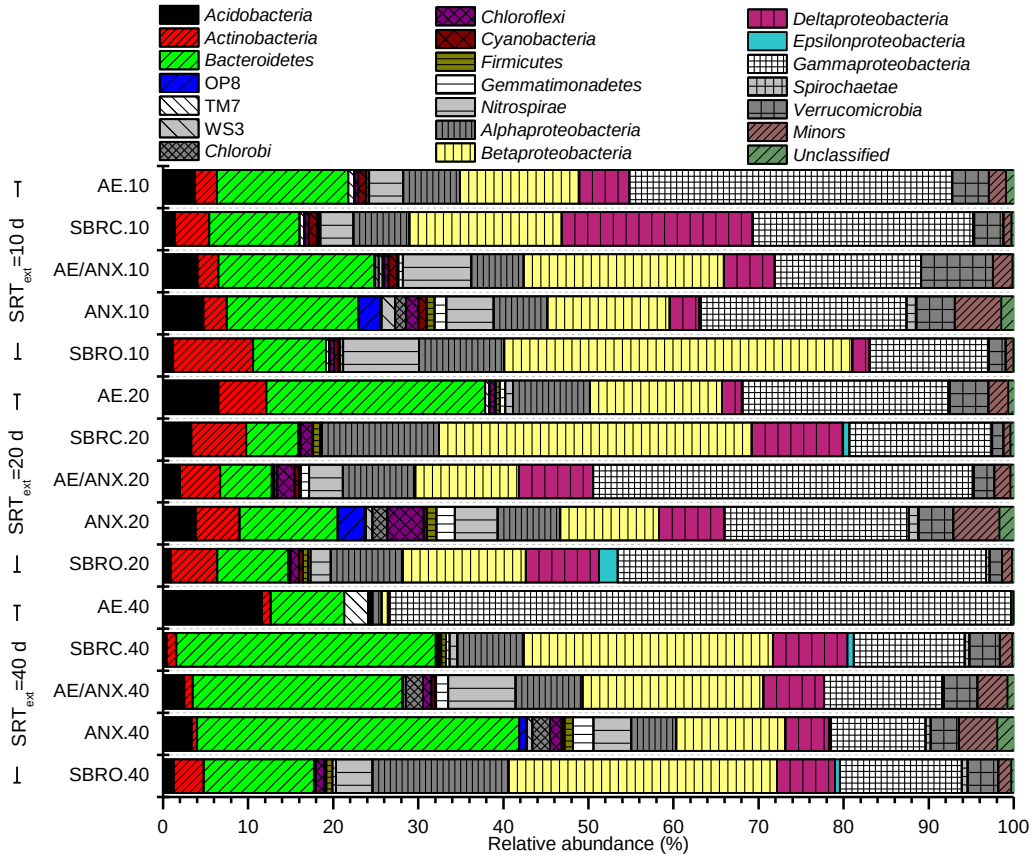

Figure 7.4. The dominant bacterial phyla (more than 1% in relative abundance) of the bacterial communities in the main SBRs and external reactors. The samples were labeled as X.Y, where X=reactor name and Y= SRT_{ext} (d). SBR_{OSA}, aerobic/anoxic reactor, and anoxic reactor of the OSA system were abbreviated as SBRO, AE/ANX, and ANX, respectively. $SBR_{control}$ and aerobic digester of the control system were abbreviated as SBRC and AE, respectively.

7.4.4.1 Comparison of SBR_{OSA} and $SBR_{control}$ microbial composition

The microbial profiles of SBR_{OSA} and $SBR_{control}$ were examined at the order level to determine their relationship with sludge reduction. The SBRs had the same SRT (10 d) yet their microbial diversity (Section 7.4.2.1) and composition (Figure 7.5) varied significantly, which implicate the influence of sludge interchange on the microbial community of the main aeration tank. *Xanthomonadales*, *Burkholdriales*, *Sphingobacteriales* and *Nitrospirales* were the four predominant orders in both SBRs. Among these, nitrifying bacteria *Nitrospirales* was consistently more abundant in SBR_{OSA} (2.4 – 8.9%; n = 15) than $SBR_{control}$ (0.1 – 3.9%; *n*=15) in all phases of the study (Figure 7.5). Other nitrifying bacteria, *Nitrosomonadales*, was a minor constituent but was also consistently more abundant in SBR_{OSA} (0.6 – 2.7%; *n*=15) than $SBR_{control}$ (0.1 – 1.2%; *n*=15). Nitrifying bacteria inherently have slow growth rate (Chon et al., 2011). The proliferation of slow-growing nitrifiers in SBR_{OSA} may contribute to the decrease of sludge yield. This is in addition to the sludge reduction due to the autolysis of sludge in the external reactors driven by the selection of distinct microbial groups (*e.g.*, hydrolysers, fermenters, and bacterial predators).

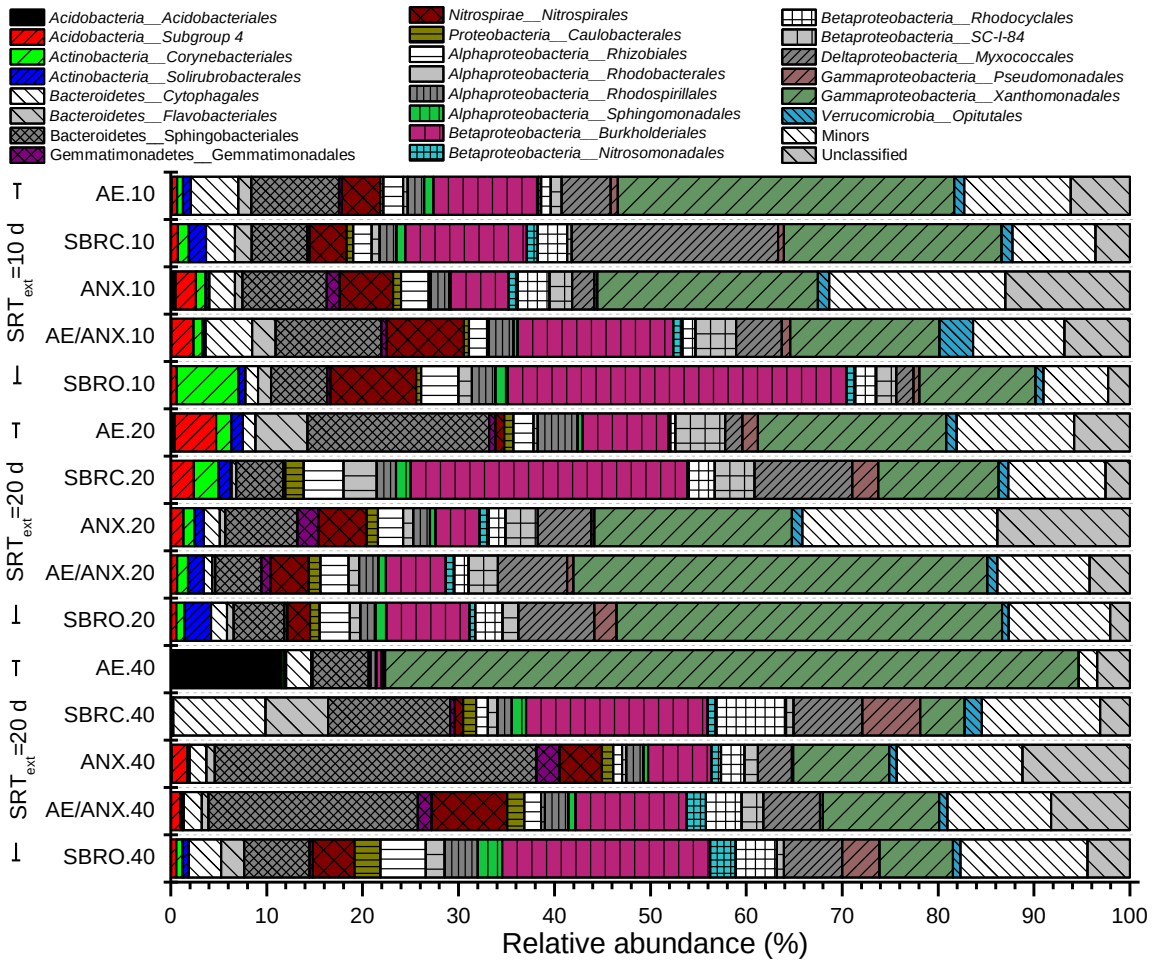

Figure 7.5. The dominant microbial orders (more than 2% in relative abundance) of the microbial communities in the main SBRs and external reactors. The samples were labelled as X.Y, where X = reactor name and Y = SRT_{ext} (d). SBR_{OSA}, aerobic/anoxic reactor, and anoxic reactor of the OSA system were abbreviated as SBRO, AE/ANX, and ANX, respectively. $SBR_{control}$ and aerobic digester of the control system were abbreviated as SBRC and AE, respectively.

A few bacterial orders were more abundant in SBR_{OSA} than $SBR_{control}$ under specific conditions (Figure 7.5). For example, *Rhodospirillales* was abundant when SRT_{ext} was 40 d (Phase I). In contrast, some microbial orders were more abundant in $SBR_{control}$ than SBR_{OSA}, including *Flavobacteriales* when SRT_{ext} was 40 d (Phase I) and *Rhdobacterales* when SRT_{ext} was 20 d (Phase II). The random appearance of these bacteria was probably due to temporal variations in domestic wastewater strength and composition (Chapter 6, Section 6.3.2).

7.4.4.2 Microbial community under oxygen-rich and -deficient conditions

Variation in the abundance of *Proteobacteria*, *Bacteroidetes*, and other major phyla were primarily influenced by redox condition or ORP level, *i.e.*, strictly aerobic (SBR_{OSA}, $SBR_{control}$, and aerobic digester), intermittent aerobic/anoxic, and strictly anoxic (Figure 7.1). This is in agreement with the results of PCoA of unweighted UniFrac (Section 7.4.3).

The phylum *Proteobacteria* had lower abundance in oxygen-deficient than oxygen-rich conditions. The relative abundance of class *β*- and *γ-Proteobacteria* decreased in the following order: aerobic (22 ± 13 and 29 ± 20%, *n*=9, respectively) > intermittent aerobic/anoxic (19 ± 6. and 25 ± 17%, respectively; *n*=3) > anoxic (12.9 ± 1.4 and 19 ± 7%, respectively; *n*=3). Previous studies observed that the *Proteobacteria* population was negatively correlated with sludge reduction. Lin *et al.* (2007) reported that the relative abundance of *β-Proteobacteria* in a gravel contact oxidation reactor (12%), a system that minimises sludge production, was lower than that of a control CAS (18%). Ning *et al.* (2006) also observed that the relative abundance of *β-Proteobacteria* decreased in the external anaerobic reactor of a laboratory-scale OSA system, and that *β-Proteobacteria* was possibly the main class that was reduced during treatment. The current study indicates that both *β*- and *γ-Proteobacteria* decayed under environmental stress in OSA. The decay of these microorganisms did not decrease the overall diversity of OSA (Section 7.4.2.2) because a greater variety of species were enriched under oxygen-deficient conditions.

The organisms that thrived under oxygen-deficient conditions included hydrolyzing and fermentative bacteria. Hydrolysing bacteria produce enzymes such as amylases, proteases, and lipases that enable the decomposition of proteins, cellulose, and other organic compounds that are not readily biodegradable under ambient conditions (Ali Shah et al., 2014). Results show that *Bacteroidetes*, which include known the known facultatively anaerobic and hydrolysing bacteria *Sphingobacteriales*, was most abundant under anoxic conditions. The relative abundance of *Bacteroidetes* increased in the following order: aerobic (14 ± 9%; *n*=9) > intermittent aerobic/anoxic (16 ± 9%; *n*=3) > anoxic (22 ± 14%; *n*=3) (Figure S5). Other hydrolyzing bacteria including members of the phyla *Chloroflexi*, such as *Gemmatimonadetes* and *Chlorobi*, were also more abundant in anoxic (2.4 ± 0.6, 2.0 ± 0.6 and 1.7 ± 0.4%, respectively; *n*=3,) than intermittently aerobic/anoxic (1.2 ± 0.8, 1.0 ± 0.5 and 0.8 ± 1.0%, respectively; n = 3) and aerobic (0.1 – 0.6%) reactors (Figure 7.4). Fermentative or acid-forming bacteria break down

products of hydrolysis and produce short-chain volatile fatty acids, alcohols, and other small organic molecules (Gerardi, 2006). Fermentative bacteria such as OP8, *Firmicutes*, WS3, and *Spirochaetae* were only found in significant abundance in the external anoxic reactor (2.2 ± 1.2, 1.0 ± 0.1, 1.0 ± 0.5 and 1.0 ± 0.3%; n=3, respectively) (Figure 7.4). The aforementioned phyla are usually found in anaerobic digesters (Foladori et al., 2010; Yang et al., 2011). *Candidate* phylum WS3 has metabolic potential to degrade a wide variety of polysaccharides and glycoproteins that are major components of extracellular polymeric substances (EPS) in activated sludge (Foladori et al., 2010). The abundance of hydrolyzing and fermentative bacteria especially in the external reactors bolster previous findings on the pertinent mechanisms of sludge autolysis in OSA, such as the destruction of volatile solids (Wang et al., 2008) and disintegration of EPS (Chon et al., 2011). Results of this study provide a micro-ecological perspective on the mechanism of sludge autolysis in the external reactors: bacteria such as β- and *γ-Proteobacteria* decrease in the external reactor, thereby producing materials that can be metabolised by hydrolyzing and fermentative bacteria for cell maintenance. The enrichment of hydrolysers and fermenters further facilitates sludge autolysis as they break down particulate and soluble organic matter. Since these bacteria play a key role in sludge reduction, their biological activity or population can be used in bioprocess monitoring. For instance, the activity of fermentative bacteria can be monitored by measuring the concentration of short-chain volatile fatty acids. This approach can reveal useful information on the progress of sludge autolysis in the external aerobic/anoxic reactor. As a well-established and relatively simple method, the measurement of short-chain volatile fatty acids can be readily implemented in full-scale plants. Meanwhile, bacterial population can be monitored through biotechnological techniques (*e.g.*, PCR). However, this approach is relatively complex and may require significant cost.

7.4.4.3 Impact of SRT_{ext} on OSA external reactors

Although redox condition was the primary factor affecting microbial diversity (Section 7.4.3), results show that SRT_{ext} also had impact on microbial community structure particularly on the population dynamics of specific bacterial groups in the external reactors. This was because SRT_{ext} directly affected the incubation period of bacteria. In the case of the aerobic/anoxic reactor, changing the SRT_{ext} also caused slight variations inthe ORP (Table 7.2) although the overall redox condition was maintained (intermittend aerobic). The current results show that the

relative abundance of the predominant order, *Xanthomonadales* (*γ-Proteobacteria*), sharply increased from 16 to 43% when SRT_{ext} was increased from 10 to 20 d, but declined to 12% when SRT_{ext} was further increased to 40 d (Figure 7.5). *Xanthomonadales* has been identified as an important denitrifying bacteria in biofilms and MBRs (Chon et al., 2011). It is possible that denitrification efficiency was in the aerobic/anoxic reactor was enhanced when *Xanthomonadales* became more abundant. This highlights the role of this particular bacterial order in the nitrogen cycle in the external reactors of OSA. Notably, members of *Xanthomonadales* can survive even in environments with minimal nitrogen (Novak et al., 2007) such as one that pervades in the external aerobic/anoxic reactor at low SRT_{ext} (< 20 d). This probably enabled *Xanthomonadales* to occupy a niche under the aforementioned conditions.

Also of note, nitrifying bacteria (*Nitrospirales* and *Nitrosomonadales*) were detected in the external aerobic/anoxic reactor under all SRT_{ext} (Figure 7.5). *Nitrospirales* accounted for 4 – 8% of the biomass and *Nitrosomonadales* accounted for 1 – 2% (Figure 7.5). The abundance of nitrifying bacteria in the current study was higher than those detected in nitrifying activated sludge of previous studies (Goel and Noguera, 2006). While *Nitrosomonadales* are well-known ammonia-oxidizing bacteria, a recent study found that *Nitrospirales* can perform complete nitrification (Datta et al., 2009). Therefore, the deficiency of nitrification/denitrification at SRT_{ext} of 40 d was not due to the loss of nitrifying species. Rather, it was because of the limitation of substrate (sCOD), corroborating the explanation in Chapter 6 (Section 6.4.3).

Specific bacteria dominated the external aerobic/anoxic reactor especially when SRT_{ext} was 10 d. For instance, the relative abundance of the order *Burkhoderiales* was 16, 6, and 12% when SRT_{ext} was 10, 20, and 40 d, respectively (Figure 7.5). This pattern correlated with high denitrification in the reactor when SRT_{ext} < 20 d (Chapter 6, Section 6.4.3) probably because this order includes denitrifying bacteria (Datta et al., 2009). However, denitrifying bacteria are notably diverse. Members of *Rhodocyclales, Pseudomonadales, Rhodospirillales, Corynebacteriale,* and *Rhizobiales* (Niu et al., 2016; Novak et al., 2007), which were all found in varying abundance in aerobic/anoxic sludge (Figure 7.5), can potentially perform denitrification. Therefore, it is possible that bacteria other than *Xanthomondales* and *Burkhoderiales* perform as active denitrifiers in the aerobic/anoxic reactor.

The families *Saprospiraceae* (14%) and *Chitinophagaceae* (7%) and the members of the order *Sphingobacteriales,* were the predominant bacteria in the external aerobic/anoxic reactor when SRT_{ext} was 40 d. Members of *Sphingobacteriales* are aerobic or facultative anaerobic bacteria (Section 7.4.2.1). Increasing the SRT_{ext} from 10 to 40 d decreased reactor ORP from approximately +50 to –150 mV when aeration was turned off (Table 7.2). This suggests that the ability of *Sphingobacteriales* to grow under anaerobic conditions (*i.e.*, low ORP) enabled them to proliferate at high SRT_{ext}.

Overall, the microbial profile of the external aerobic/anoxic reactor reinforces the observation that high nitrification/denitrification efficiency was favoured at SRT_{ext} of 10 and 20 d (Chapter 6, Section 6.4.3). The increase in the population of denitrifying bacteria at the optimum SRT for sludge reduction (20 d) further emphasises that denitrification is a key reaction driving sludge reduction in the external reactors of OSASpecifically, denitrification helps ensure that destroyed volatile solids are converted into inert products (Chapter 6, Section 6.4.3).

It was demonstrated in Chapter 6 (Section 6.4.3) showed that increasing SRT_{ext} from 10 to 20 d enhanced volatile solids destruction in the external anoxic reactor, but further increasing SRT_{ext} to 40 d did not result in further solids destruction. The microbial profile of the anoxic reactor further helped in elucidating the impact of SRT_{ext} on sludge autolysis (Figure 7.5). *Xanthomonadales* (18 ± 7%; n = 3) and *Sphingobacteriales* (17 ± 15%; n = 3) were the predominant orders in the external anoxic reactor at all SRT_{ext} (Figure 7.5). The relative abundance of nitrifying (*Nitrosomonadales* = 1.0 ± 0.0% and *Nitrospirales* = 5.0 ± 0.6%; n = 3) and denitrifying (*Burkholderiales* = 5.7 ± 1.0%; n = 3) bacteria were similar at different SRT_{ext} (Figure 7.5). The population of these bacteria were stable because the ORP (<400 mV) of the external anoxic reactor was maintained even though SRT_{ext} was varied.

Predatory bacteria were especially enriched in the external anoxic reactor, and their population dynamics correlated with the efficiency of cell lysis in the external anoxic reactor. The abundance of predatory bacteria *Myxobacteriales* and *Bdellovibrio* in the anoxic reactor was at the maximum at SRT_{ext} of 20 d, was the optimum SRT_{ext} for cell lysis (Section 6.4.3). *Myxobacteria,* which are Gram-negative bacteria that are usually found in soils and aquatic environments, secrete metabolites to damage the cell wall of other bacteria (Zhou et al., 2014). *Bdellovibrio,* is a genus of Gram-negative obligate predators that attach to other Gram-negative

bacteria and utilise the macromolecules of the prey cell to growth (Da-Zhi et al., 2016; Ferrera and Sánchez, 2016), was the highest when SRT_{ext} was 20 d (0.61%; n = 3) (data not shown). Niu *et al.* (2014) also found *Bdellovibrio* and similar predatory bacteria in an oxygen-deficient tank attached to CAS to achieve sludge reduction. In this study, increasing the SRT_{ext} beyond 20 d did not cause further improvement to cell lysis, possibly because the remaining biomass was able to survive using lysates as substrate under oxygen-deficient conditions. The abundance of predatory bacteria was at the maximum at SRT_{ext} of 20 d and slightly declined at SRT_{ext} of 40 d, suggesting that their population was stable at $SRT_{ext} \geq 20$ d. The correlation between predatory bacterial population and cell lysis efficiency indicates that these microorganisms play a significant role in volatile solids destruction in the external anoxic reactor. Therefore, the enrichment and activity of predatory bacteria in the external reactors contribute to the overall sludge reduction in OSA.

The maximum abundance of certain hydrolyzing bacteria was observed at the SRT_{ext} of 20 d (Figure 7.5). This pattern was especially observed in *Chloroflexi*, which are significant hydrolyzing bacteria in wastewater systems (Yadav et al., 2014); they had an abundance of 1.5, 4.3, and 1.4% at SRT_{ext} of 10, 20, and 40 d, respectively. This indicates that in addition to predatory bacteria, hydrolyzing bacteria were essential to the process of sludge autolysis in the external anoxic reactor.

It was previously observed that orthophosphate accumulated in the anoxic reactor regardless of SRT_{ext}, which indicated that EBPR did not occur (Section 6.4.3). Indeed, the dominant bacteria that are known to accumulate phosphorous (*Acinetobacter*) (Gerardi, 2006) were not identified in the anoxic reactor. Minor bacteria associated with phosphorous accumulation (e.g., *Actinobacteria* and *α-Proteobacteria*) were found (Figure 7.4), but they possibly had limited capacity for phosphorous uptake under substrate-deficient conditions (Crocetti et al., 2000).

7.4.4.4 Impact of SRT_{ext} on the control aerobic digester

Similar to the external reactors of OSA, the aerobic digester was under substrate-deficient conditions (Section 7.4.2.3), however, minimal sludge autolysis occurred (Chapter 6, Section 6.4.2). In this chapter, the bacterial profile of the aerobic digester was analyzed to determine the impact of substrate deficiency on sludge with continuous supply of oxygen. This provides a point

of comparison for assessing the synergistic effect of withholding both substrate and oxygen from sludge, as in the case of the external reactors of OSA.

PCoA showed that the microbial composition and structure of $SBR_{control}$ and the aerobic digester were highly similar throughout the operating period (Section 7.4.3) except when SRT_{ext} was 40 d (Phase I). During this time, the low pH (< 5.5) of the aerobic digester affected its microbial community. $SBR_{control}$ and aerobic digester had comparable microbial composition probably because the two reactors had similar DO concentration (> 5 mg/L). Also, the configuration of the control system, *i.e.,* the aerobic digester received sludge solely from $SBR_{control}$, ensured that the microbial community of the latter reactor was dependent on the former. However, a few bacteria had varying population in $SBR_{control}$ and aerobic digester. For example, the percent composition of the orders *Burkholderiales*, *Rhodocyclales,* and *Myxococcales* in the aerobic digester (7 ± 6, 7 ± 6 and 2.3 ± 2.6%, respectively; n = 3) was markedly lower than that of $SBR_{control}$ (21 ± 8, 20 ± 8 and 13 ± 8%; n = 3) (Figure 7.5). There is little information about the behavior of these bacteria, which are all obligate aerobes (Stadler and Love, In press), under aerobic digestion. Their population probably diminished due to lack of readily biodegradable substrate in the aerobic digester. On the contrary, the percent composition of orders *Xanthomonadales* and *Sphingobacteriales* in the aerobic digester (42 ± 27 and 11 ± 7%, respectively; n = 3) was higher than that of $SBR_{control}$ (13 ± 9 and 8 ± 4%, respectively; n = 3). The aforementioned bacteria were also found at significant concentration in the external reactors of OSA (Section 7.4.4.2), suggesting that they can flourish despite the starvation conditions. These results indicate that although DO concentration is a key factor affecting microbial composition in sludge, the availability of substrate also contributes to shifts in microbial community structures.

Xanthomonadales (35 – 70%) was the most abundant order in the aerobic digester at all SRTs (Figure 7.5). These bacteria were one of the four major orders in the SBRs (Section 7.4.4.1) and also the predominant order in the external aerobic/anoxic reactor of OSA (Section 7.4.4.2). Aside from the fact that *Xanthomonadales* thrive at both high and low nitrogen loads under aerobic condition (Spietz et al., 2015), little is known about the growth pattern of this order at varying redox regimes. However, current findings suggest that *Xanthomonadales* can survive under substrate-deficient conditions.

Nitrification was inhibited in the aerobic digester (Chapter 6, Section 6.4.2). The current findings confirm that certain bacteria that perform nitrification (*e.g.*, *Nitrospirales*) and nitrogen-fixation or conversion of molecular nitrogen to ammonium ions (*Rhiziobiales*) had fluctuating and low abundance (0 – 5%) at all SRT_{ext}. Nitrifying bacteria are highly sensitive to various environmental conditions (*e.g.*, temperature, pH, alkalinity, organic compounds). In this study, it is possible that the pH of the aerobic digester (6.2 – 6.9) was too low for specific nitrifying bacteria to grow. For instance, pH 6.5 – 8.5 is the ideal growth range for genus *Nitrobacter*, the bacteria that convert nitrite to nitrate (Yadav et al., 2014). Possibly, the abundance of its parent order, *Rhiziobiales*, was very low (0 – 2%) in the aerobic digester due to low pH.

Some bacterial orders became more abundant when SRT_{ext} was increased from 10 to 20 d, and then declined when SRT_{ext} was increased to 40 d. These included *Sphingobacteriales*, *Flavobacteriales*, Subgroup 4, and SC-I-84 (Figure 7.5). Notably, with the exception of *Spingobacteriales* (5.8%), the abundance of the aforementioned orders was nearly zero at SRT_{ext} of 40 d. *Spinghobacteriales* are hydrolyzing bacteria (Spietz et al., 2015) that were also found in the external reactors of OSA (Section 3.4.3). *Flavobacteriales* are other hydrolyzing bacteria that can break down carbohydrates such as starch and gelatin (Xing et al., 2016).

The microbial diversity of the aerobic digester peaked at SRT_{ext} of 20 d, and then sharply decreased at SRT_{ext} of 40 d (Section 3.2.3) with *Xanthomonadales* accounting for 72% of the community abundance. *Xanthomonadales*, as discussed earlier, are resilient bacteria that can survive under environmental stress involving oxygen and substrate deficiency (Figure 7.5). Another order that became predominant at SRT_{ext} of 40 d was *Acidobacteriales* (11%), which could survive under highly acidic conditions (Pijuan et al., 2009). In this study, the pH of the aerobic digester ranged from 5.2 – 6.7. The periods of low pH (< 5.5) probably allowed this order to proliferate. Nonetheless, as mentioned in Section 3.2.3, the microbial diversity of the aerobic digester at this phase of the study was extremely low, so potential errors in sampling cannot be ruled out completely.

Generally, the patterns observed in the aerobic digester (*i.e.*, lack of nitrification/denitrification and sludge autolysis) were corroborated by its microbial diversity (Section 7.4.2.3) and composition. The microbial profile of the aerobic digester also showed both substrate- and oxygen-deficient environments must be fulfilled to facilitate sludge autolysis in external reactors.

7.5 CONCLUSION

The microbial diversity and composition of a laboratory-scale OSA fed with real wastewater were determined. Constrained PCoA of unweighted Unifrac distances demonstrated that redox condition was the most important factor affecting microbial diversity. Microbial diversity in reactors increased in the following order: aerobic < intermittent aerobic/anoxic < anoxic. Generally, SBR_{OSA} had greater abundance of slow-growing nitrifying bacteria, which may explain the lower sludge yield compared to $SBR_{control}$. A wider range of microorganisms such as hydrolyzing, fermentative, denitrifying and predatory bacteria proliferated in the external reactors of OSA. Predatory and denitrifying bacteria were most abundant the external reactors of OSA at SRT_{ext} of 20 d (the optimum condition for sludge reduction). Predators probably facilitated sludge autolysis, while denitrifiers probably played a key role in converting destroyed volatile solids into inert forms.

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CHAPTER 8: Fate of trace organic contaminants (TrOCs) in the oxic-settling-anoxic (OSA) process

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8.1 INTRODUCTION

Trace organic contaminants (TrOCs) are pharmaceuticals, pesticides, personal care products, hormones, and other compounds that are ubiquitously found in trace concentrations in the environment. TrOCs can damage the endocrine system, which govern the physiological development and reproduction of animals and humans. Some TrOCs in wastewater are resistant to microbial degradation. The fate of TrOCs during wastewater treatment is dependent on chemical properties. For example, non-biodegradable and hydrophilic compounds are unaffected by wastewater treatment and thus persists in the effluent in their original form. Meanwhile, non-biodegradable and hydrophobic TrOCs bind to the surface of sludge flocs and accumulate in biosolids (Semblante *et al.*, 2015a). The occurrence of TrOCs in either effluent or biosolids could result in the emission of these contaminants in receiving water bodies, agricultural land, or groundwater (Clarke and Cummins, 2015). Because of this, research efforts have been devoted to determine the fate of TrOCs in full-scale wastewater treatment systems (Janssen *et al.*, 2015; Phan *et al.*, 2015; Trinh *et al.*, 2016). TrOC sorption and biodegradation are affected by operating conditions such as redox (*e.g.*, aerobic, anoxic, or anaerobic), SRT, and others (Semblante *et al.*, 2015a). However, the fate of TrOC in OSA has not been investigated.

This study aims to determine the sorption and biodegradation of TrOCs in OSA operated using real wastewater. The TrOC concentrations in the effluent and sludge of an OSA system were compared to that of a control system to gain insight on the effects of sludge interchange between different redox regimes on the fate of TrOCs. Furthermore, the fate of TrOCs was determined at different external reactor SRT (SRT_{ext}; defined in Chapter 3, Section 3.2.3). The findings of this chapter are relevant to the assessment of the TrOC emission of OSA and in the future development of TrOC mitigation or treatment approaches.

8.2 HYPOTHESIS

- The fate of TrOCs in OSA may be impacted by redox condition and SRT_{ext}.
- The emission of the OSA system may be different from that of the control system.

8.3 MATERIALS AND METHODS

8.3.1 Reactor configuration and operation

Details on the configuration and operation of the laboratory-scale OSA (SBR_{OSA} attached to external aerobic/anoxic and anoxic reactors) and control ($SBR_{control}$ attached to single-pass aerobic digester) systems are described in Chapter 3 (Section 3.2).

The SRT_{ext} of both systems was varied as described in Chapter 6 (Section 6.3.1). In the OSA system, this was performed by adjusting volume of sludge discarded from the aerobic/anoxic reactor (q_3) (Chapter 3, Section 3.2.1). In the control system, this was performed by adjusting the volume of sludge discarded from the aerobic digester (Q_{out}) (Chapter 3, Section 3.2.2). The SRT of SBR_{OSA} and $SBR_{control}$ (SRT_{SBR}; defined in Chapter 3, Section 3.2.3) was maintained at 10 d. The SIR of the OSA system was maintained at 11%. $FeCl_2$ was not added to the influent. The SIR of the OSA system was maintained at 11%. $FeCl_2$ was not added to the influent. A summary of the experimental phases is described in Table 7.1 (Chapter 7).

The average conditions in the OSA and control system reactors at different experimental phases in this chapter are summarised in Table 7.2 (Chapter 7).

8.3.2 Domestic sewage

Domestic unsettled sewage with properties described in Chapter 6 (Section 6.3.2) was collected from the beginning of the primary sedimentation channel of Wollongong WWTP fortnightly and stored at 4 ℃ prior to use.

8.3.3 Analytical techniques

8.3.3.1 Wastewater and sludge analysis

The solids concentration of sludge were measured as described in Chapter 3 (Section 3.5.1.1 and 3.5.1.2), respectively. The solids concentration, TOC/TN, sCOD concentration, ammonia concentration, and phosphate concentration of wastewater were measured as described in Chapter 3 (Section 3.5.1.1 to 3.5.1.5). The DO concentration, pH, and ORP of wastewater and sludge were measured as described in Chapter 3 (Section 3.5.1.9).

8.3.3.2 TrOC extraction and analysis

Duplicate measurements of the TrOC concentration of the influent (domestic sewage), effluent, and sludge were obtained at the end of each experimental phase (SRT_{ext}=10, 20, and 40 d), which corresponded to summer (December 2015), spring (October 2015), and winter (July 2015) seasons (Table 8.1). The list of TrOCs that were analysed at each operation period, along with their chemical properties and detection limits, are listed in Table B.3. All samples were prepared as described in Chapter 3 (Section 3.5.4.1). The samples, which were mixed with surrogate solution containing isotopically labelled standards used to determine sample recovery, were further concentrated and purified through SPE as described in Chapter 3 (Section 3.5.4.2). TrOC concentration was determined using HPLC-TQMS as described in Chapter 3 (Section 3.5.4.3).

Table 8.1. Summary of (a) TrOC sampling and (b) sludge reduction by OSA at different experimental phases in this chapter. The SRT_{ext} was varied (10-40 d) while the SRT_{SBR} was maintained 10 d, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage). The tCOD values are the average ± standard deviation where *n* = number of measurements.

Experimental Phase	**SRT_{SBR}**	**SRT_{ext}**	**TrOC sampling season**	**Influent tCOD (mg/L)**	**Sludge yield (g MLVSS/g tcOD)**		**Sludge reduction (%)**
					SBR_{OSA}	*$SBR_{control}$*	
I	10	10	Summer	527±154 (n=19)	0.19	0.16	0
II	10	20	Spring	478±254 (n=12)	0.09	0.14	35
III	10	40	Winter	491±194 (n=11)	0.13	0.13	16

8.3.4 Calculations

8.3.4.1 Sludge reduction

Sludge reduction was calculated as the difference in sludge yield of SBR_{OSA} and $SBR_{control}$. In this study, sludge yield *Y* is defined as the cumulative sludge produced in terms of MLVSS (*P*)

over the cumulative substrate consumed in terms of tCOD (*C*). The detailed calculation of sludge yield is described in Chapter 3 (Section 3.4).

8.3.4.2 TrOC concentration

To analyse the biodegradation and sorption of TrOCs under aerobic/anoxic treatment, the TrOC concentration of sludge (in ng/L) going in to the reactor ($Y_{in\text{-}aerobic/anoxic}$) was estimated based on sludge flows from SBR_{OSA} ($Y_{SBR_{OSA}}$) and anoxic reactor (Y_{anoxic}):

$$Y_{in-aerobic/anoxic} = Y_{SBR_{OSA}} + Y_{anx} \quad \textbf{Equation 8.1}$$

$$Y_{in-aerobic/anoxic} = \frac{[(A_{SBR_{OSA}} + S_{SBR_{OSA}} \times MLSS_{SBR_{OSA}}) \times q_1 + (A_{anoxic} + S_{anoxic} \times MLSS_{anoxic}) \times q_4]}{(q_1 + q_4)} \quad \textbf{Equation 8.2}$$

Where A and S are the aqueous and solid phase TrOC concentration of sludge, MLSS was the sludge concentration, q_1 is the flow rate of sludge from SBR_{OSA} to aerobic/anoxic reactor (Chapter 3, Figure 3.1a), and q_4 is the flow rate of sludge from anoxic to aerobic/anoxic reactor (Chapter 3, Figure 3.1a).

Likewise, the TrOC concentration of sludge going in to the anoxic reactor was estimated based on sludge flow from the aerobic/anoxic reactor ($Y_{aerobic/anoxic}$):

$$Y_{in-anoxic} = Y_{aerobic/anoxic} \quad \textbf{Equation 8.3}$$

$$Y_{in-anoxic} = A_{aerobic/anoxic} + S_{aerobic/anoxic} \times MLSS_{aerobic/anoxic} \quad \textbf{Equation 8.4}$$

Notably, the flow rate of sludge from the aerobic/anoxic to the anoxic reactor (q_3) was equal to the rate at which sludge was withdrawn from the anoxic reactor (q_4+q_5) (Chapter 3, Figure 3.1a).

The TrOC concentration of sludge going in to the aerobic digester was also estimated based on sludge flow from $SBR_{control}$ (Chapter 3, Figure 3.1b):

$$Y_{in-aerobic} = Y_{SBR_{control}} \quad \textbf{Equation 8.5}$$

$$Y_{in-aerobic} = A_{SBR_{control}} + S_{SBR_{control}} \times MLSS_{SBR_{control}}$$ **Equation 8.6**

8.4 RESULTS AND DISCUSSION

8.4.1 Sludge reduction by OSA

Sludge reduction by OSA at different SRT_{ext} has been discussed in Chapter 6 (Sections 6.4.2 and 6.4.3). Briefly, increasing SRT_{ext} from 10 to 20 d enhanced sludge autolysis in the external reactors. However, increasing SRT_{ext} from 20 to 40 d did not increase sludge autolysis further. Additionally, maintaining relatively low SRT_{ext} (10 and 20 d) facilitated the conversion of destroyed sludge into inert products through denitrification and nitrification reactions. Therefore, an intermediate SRT_{ext} (20 d) favoured sludge reduction in OSA (Chapter 6, Sections 6.4.2 and 6.4.3). Furthermore, regardless of the SRT_{ext}, SBR_{OSA} and $SBR_{control}$ effluent had similar tCOD and ammonia concentration (Chapter 6, Sections 6.4.2). This suggested that OSA did not affect the overall wastewater treatment efficiency of the main aeration tank (SBR_{OSA}).

8.4.2 TrOC concentration in the influent

The sampling campaigns at different SRT_{ext} fell at different seasons (Table 8.1). Sludge reduction was estimated by comparing the performance of $SBR_{control}$ and SBR_{OSA} during a certain operation regime, and thus was not affected by variation in influent wastewater characteristics. On the other hand, sampling at different seasons helped to obtain a comprehensive profile of TrOCs in the influent (domestic sewage) in the study site. A total of 52 TrOCs were detected throughout the operating period (Figure 8.1). Thirty-four (34) out of 45 target TrOCs were detected during the winter sampling campaign, whereas 45 out of 60 target TrOCs were detected during the spring and summer sampling campaigns (Supplementary Figure S5a). The TrOCs had a wide range of concentrations (10-100,000 ng/L). The majority of the detected TrOCs are consumed and/or secreted by humans on a daily basis, such as food products, pharmaceuticals, personal care product ingredients, and hormones (endogenous and synthetic). The food products included artificial sweeteners and caffeine. The pharmaceuticals included antibiotics, beta-blockers, nonsteroidal anti-inflammatory drugs, tranquilisers, anticonvulsants, and antidepressants (Table B.3). Additionally, pesticides and industrial chemicals were detected in the influent, albeit at relatively low concentrations. Among the seven target pesticides, only

diazinon (an insecticide), phenylphenol (a fungicide), simazine (a herbicide) and diuron (a herbicide) were found in the influent at a concentration range of 5-150 ng/L (Figure 8.1). Chemicals with industrial applications, such as tris-2-chloroethyl phosphate (TCEP, flame retardant), bisphenol A (an intermediate used in manufacturing of plastics and epoxy resins), 4-tert-octylphenol (a surfactant), and nonylphenol (a surfactant), were also found in the influent. TCEP and bisphenol A had a concentration range of 500-1,000 ng/L, whereas 4-tert-octylphenol and nonylphenol only had a concentration range of 5-70 ng/L (Figure 8.1).

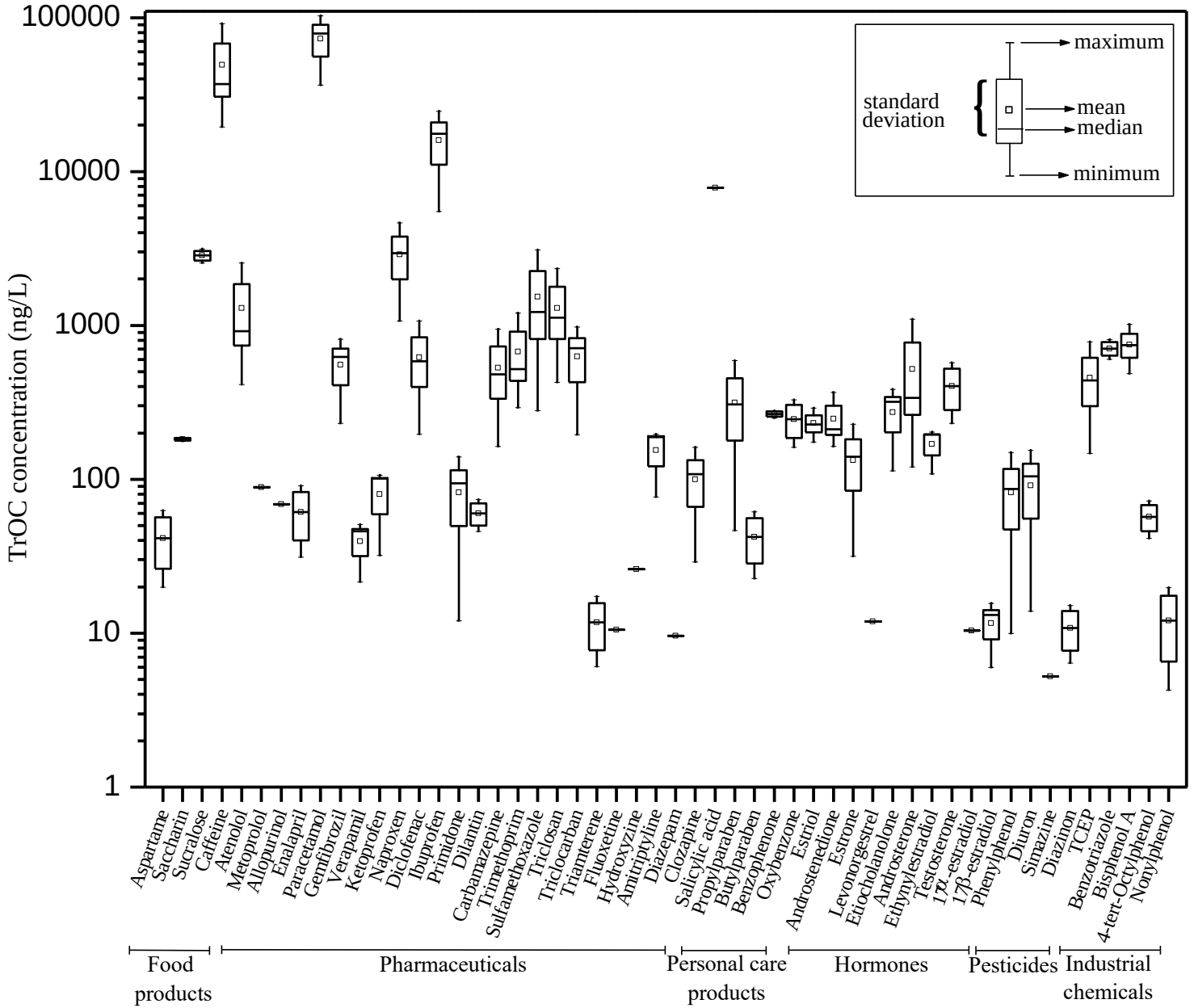

Figure 8.1. TrOCs detected in the influent (domestic sewage). The values are the average of six measurements (n=6).

TrOC concentration in the influent increased with season in the following order: winter<summer<spring (Figure A.7). The maximum concentration of some TrOCs (particularly food products, pharmaceuticals, and personal care products) was approximately 100,000 ng/L in summer and spring, whereas it was only approximately 40,000 ng/L in winter. Because TrOC concentrations were low compared to other organic constituents of wastewater, TrOCs had negligible contribution to tCOD. Indeed, results show that TrOC concentration had no relationship with influent strength in terms of tCOD, which had nominal variation in the entire experimental period (Table 8.1).

It is noteworthy that several endogenous hormones and metabolic products (estriol, androstenedione, etiocholanolone, and 17β-estradiol) had similar concentration in domestic sewage in all seasons (Figure 8.2a). There was also similar concentration of ethinylestradiol, a synthetic estrogen that is commonly used in contraceptive pills and hormone replacement therapy, in all seasons (Figure 8.2a). The human secretion of these compounds is probably unaffected by seasonal changes. The study of Trinh *et al.*, (2016) also observed that the influent concentration of hormones and other TrOCs in Bega Valley, Australia had similar concentration regardless of the season.

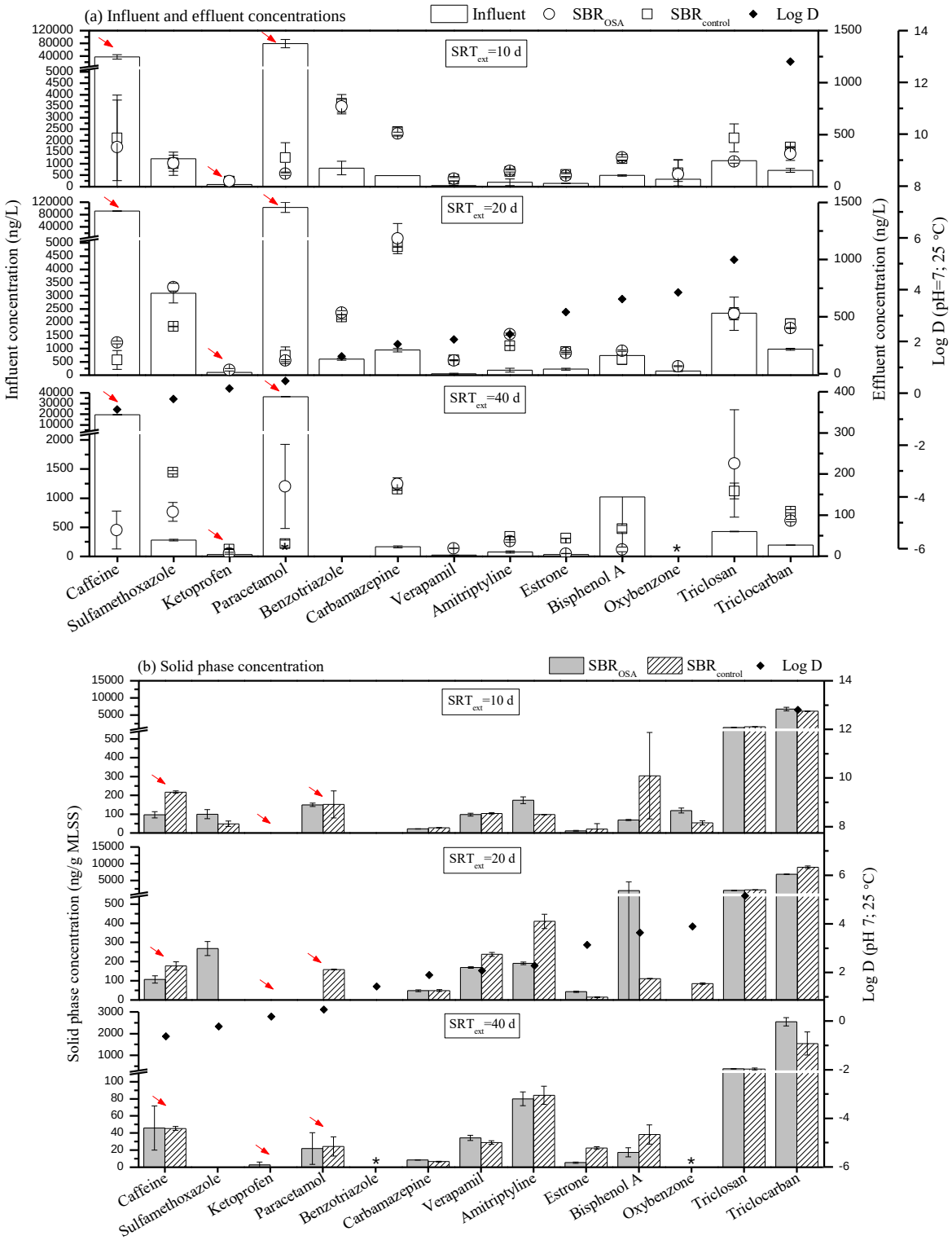

Figure 8.2. Concentration of selected TrOCs in the (a) influent and effluent, and (b) solid phase of sludge of SBR_{OSA} and $SBR_{control}$ when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average of two measurements (*n*=2). The asterisks represent contaminants that were not analysed in a particular sampling campaign. The arrows (→) denote contaminants that were highly biodegraded in the SBRs.

Among the detected TrOCs contaminants, the highest influent concentration (>5,000 ng/L and >1,000 ng/L during spring/summer and winter seasons, respectively) was observed for salicylic acid, caffeine, paracetamol, and ibuprofen regardless of the season (Figure 8.1). Caffeine is a stimulant added to many types of beverages, food, and pharmaceuticals, while the other compounds are key ingredients in over-the-counter ointments and/or orally-ingested medicines (Luo *et al.*, 2014). The aforementioned compounds were also found in high concentrations in the wastewater of other parts of Australia (Phan *et al.*, 2015; Trinh *et al.*, 2016) probably because of the similarity in human consumption in these areas. For instance, paracetamol is one of the most highly consumed drugs in terms of daily dose/thousand population/day in Australia as of 2014 (PBS/DH, 2014).

8.4.3 TrOC concentration in the SBR effluent

Aerobic treatment can result in TrOC biodegradation and mineralisation, but CAS is not tailor-fitted for TrOC removal and therefore certain compounds may persist in the effluent or sludge (Semblante *et al.*, 2015a). The concentrations of all detected TrOCs in the influent, effluent, and solid phase of sludge are presented in Figure A.7. The concentrations of selected TrOCs representing highly biodegradable (caffeine, ketoprofen, and paracetamol), partially biodegradable (sulfamethoxazole and bisphenol A), and poorly biodegradable (benzotriazole, carbamazepine, verapamil, amitriptyline, estrone, oxybenzone, triclosan, and triclocarban) contaminants are presented in Figure 8.2. Among the selected non-biodegradable compounds, benzotriazole, carbamazepine, and estrone (Figure 8.2a) were detected mostly in the effluent whereas verapamil, amitriptyline, triclosan and triclocarban (Figure 8.2b) were detected mostly on the solid phase of sludge. Furthermore, the concentration of caffeine, benzotriazole, estrone, and triclosan in the effluent (Figure 8.2) exceeded threshold concentrations set for recycled water in Australia.

8.4.3.1 SBR_{OSA} effluent

Hydrophilic TrOCs (log D<3; pH=7; 25 ºC) such as caffeine, ketoprofen, paracetamol (Figure 8.2), naproxen, ibuprofen, estriol, androstenedione, and propylparaben (Figure A.7) were highly biodegraded in SBR_{OSA}, *i.e.*, the amounts in both the effluent and sludge solid phase were lower than the influent load by more than 80%. This corroborates previous findings that the

aforementioned compounds are highly biodegraded by aerobic treatment (Radjenović *et al.*, 2009; Tadkaew *et al.*, 2011; Trinh *et al.*, 2016). On the other hand, hydrophilic TrOCs such as benzotriazole, carbamazepine (Figure 8.2), TCEP, sucralose, trimethoprim, dilantin, diclofenac, diuron, and diazepam (Figure A.7) were non-biodegradable and mostly found in the effluent. The concentrations of the aforementioned compounds in the influent and effluent were comparable (*e.g.*, benzotriazole and caffeine in Figure 8.2a) but the amount detected in the sludge solid phase was very low (<5% of the influent mass load) (Figure 8.2b). With the exception of sucralose, all the aforementioned compounds possess electron-withdrawing groups (EWG) that decrease the electron density of the aromatic ring and consequently inhibit electrophilic attack by oxygenases, which is the potential first step in aerobic biodegradation (Hai *et al.*, 2011; Tadkaew *et al.*, 2011). There was also little or no biodegradation of the aforementioned compounds in CAS (Barceló and Petrovic, 2008; Suarez *et al.*, 2010; Pasquini *et al.*, 2014) and membrane bioreactor (MBR) (Tadkaew *et al.*, 2011; Phan *et al.*, 2015). Sucralose, a noncaloric artificial sweetener, is especially designed to be chemically stable and resist human digestion. Low biodegradation of sucralose has been observed in aerobic batch tests with and without the presence of primary substrate (sucrose) (Torres *et al.*, 2011).

Several hydrophilic TrOCs were only partially biodegraded in SBR_{OSA}. Sulfamethoxazole (Figure 8.2), atenolol, aspartame, salicylic acid, saccharin, primidone, triamterene, and gemfibrozil (Figure A.7) were partially removed from the influent, but had varying concentration in SBR_{OSA} effluent and sludge solid phase at different sampling campaigns. Among these compounds, only saccharin and sulfamethoxazole have EWGs in the form of amide and sulfonamide (Table B.3), respectively, which prevents the biodegradation of the compound. The rest have electron-donating groups (EDG) (Table B.3) that enrich the electron density of the aromatic ring and facilitate electrophilic attack (Tadkaew *et al.*, 2011). However, the complete biodegradation of the EDG-bearing compounds did not occur probably due to the relatively low SRT (10 d) of SBR_{OSA}. Clara *et al.* (2005) observed that SRT of the aeration tank must be greater than 10 d in order to have sufficient biodegradation of pharmaceuticals and personal care products. Increasing SRT increases the diversity of microorganisms and metabolic pathways, and thus it can enhance the biodegradation of certain TrOCs (Semblante *et al.*, 2015a). A full-scale aerobic/anoxic sludge reactor operated under the same SRT as this study (10 d) also had partial biodegradation of atenolol and gemfibrozil (Radjenović *et al.*, 2009). On the contrary, a pilot-

scale aerobic/anoxic MBR operated at higher SRT (25 d) than this study had high biodegradation of atenolol, gemfibrozil, salicylic acid, and amitriptyline (Phan *et al.*, 2015). These findings suggest that the biodegradation of some TrOCs could be improved by increasing SRT of the main aeration tank (SBR_{OSA}). It is noteworthy that triamterene was only partially biodegradable in either SBR_{OSA} (SRT=10 d) or aerobic/anoxic MBR (SRT=25 d) (Phan *et al.*, 2015), which may suggest that the biodegradation of this compound is not affected by SRT. Furthermore, the two artificial sweeteners that were partially biodegraded in SBR_{OSA} (aspartame and saccharin) had high biodegradation in aerobic/anoxic processes (Tran *et al.*, 2014; Tran *et al.*, 2015). Previous batch experiments showed that aspartame and saccharin were co-metabolised by nitrifying sludge in the presence of primary substrate such as ammonium and acetate (Tran *et al.*, 2014). It is possible that the microbial consortia and substrate in SBR_{OSA} were not able to facilitate co-metabolic biodegradation of these artificial sweeteners.

Two hydrophilic TrOCs, namely verapamil and amitriptyline (Figure 8.2), had low biodegradation and high sorption on sludge. The fate of these compounds is SBR_{OSA} sludge is discussed in more detail in Section 8.4.4.1.

Among the hydrophobic TrOCs (log D>3), phenylphenol, levonorgestrel, butylparaben, diazinon, etiocholanolone, androsterone, ethynylestradiol, 17-α-estradiol, and 17-β-estradiol were mostly biodegraded as evidenced by their low concentration in both SBR_{OSA} effluent and sludge (Figure A.7). Except for diazinon, the aforementioned compounds were also highly biodegraded in previous studies (Tadkaew *et al.*, 2011; Phan *et al.*, 2015; Trinh *et al.*, 2016). Diazinon is an aromatic organophosphate that is usually recalcitrant during aerobic treatment (Luo *et al.*, 2014), but it has been found to break down in sludge acclimatised to compounds having similar chemical structure (*e.g.*, chlorpyrifos) (Deng *et al.*, 2015). It is possible that the biomass of $SBR_{OSA,}$ which continuously received domestic sewage that contained agricultural chemicals and pesticides, had acclimatised to diuron. Bisphenol A was the only partially biodegraded in SBR_{OSA} (Figure 8.2). Bisphenol A had varying biodegradation and sorption rates at each sampling campaign, possibly due to the low SRT of SBR_{OSA} (10 d). Previous studies showed that bisphenol A was rapidly sorbed and biodegraded in activated sludge especially at SRT of more than 30 d (Tadkaew *et al.*, 2011). The biodegradation of bisphenol A could potentially be enhanced if SRT of SBR_{OSA} were increased.

The other hydrophobic TrOCs such as estrone, oxybenzone, triclosan, triclocarban (Figure 8.2), benzophenone, clozapine, 4-tert-octylphenol, and nonylphenol (Figure A.7) were non-biodegradable. Among the aforementioned compounds, only estrone remained in significant concentration in SBR_{OSA} effluent (Figure 8.2). A previous study have shown that estrone and its residues have greater tendency to remain in the effluent than to partition in sludge possibly because of its moderate hydrophobicity (log D = 3.13; pH 7; 25 ºC) (Verlicchi *et al.*, 2012). Estrone has potential to accumulate in the effluent through the oxidation of 17β-estradiol or partial conjugation of other hormones by the β-glucuronidase enzyme produced by fecal bacteria (D'Ascenzo *et al.*, 2003). The rest of the compounds (triclosan, triclocarban, and others) had propensity to accumulate in the sludge solid phase, and they are discussed in more detail in Section 3.4.1.

8.4.3.2 Comparison of SBR_{OSA} and $SBR_{control}$ effluent

The effect of OSA on effluent quality is an important criterion to evaluate the effectiveness of OSA as a sludge reduction strategy. Previous studies based on synthetic (Goel and Noguera, 2006) and real wastewater (Semblante *et al.*, 2016) showed that OSA did not have deleterious effect on the organic or nutrient removal of CAS. In this study, the impact of OSA on the fate of TrOCs was assessed by comparing the effluents of SBR_{OSA} and $SBR_{control}$. Similar to SBR_{OSA}, $SBR_{control}$ had constant SRT (10 d) throughout the entire experimental period (Table 8.1). Therefore the effluent of $SBR_{control}$ was used as the baseline for comparison of SBR_{OSA} effluent TrOC concentrations.

Results generally show that there was minimal difference (<10-20%) between the TrOC levels of the two effluents. This suggests that OSA did not impact TrOC biodegradation and sorption in activated sludge. The two SBRs had an identical set of biodegraded and recalcitrant compounds (Section 8.4.3.1). Some TrOCs were noticeably higher or lower (*i.e.*, more than 30% difference) in SBR_{OSA} than in $SBR_{control}$ effluent at a specific SRT_{ext} only (Table 8.2). These include biodegradable (*e.g.*, caffeine, ketoprofen, naproxen, paracetamol, ibuprofen, estriol, and androsterone), partially biodegradable (*e.g.*, atenolol, aspartame, salicylic acid, sulfamethoxazole, gemfibrozil, and bisphenol A), and non-biodegradable contaminants (*e.g.*, 4-tert-octylphenol, oxybenzone, nonylphenol, and triclosan). However, the variations were not consistently observed at different SRT_{ext} (Table 8.2). Therefore, these variations could not be

attributed to SRT_{ext} but rather on changes in influent concentration or biodegradation rates (especially for partially and non-biodegradable TrOCs).

Table 8.2. TrOCs with notable variation (more than 30% difference) in SBR_{OSA} and $SBR_{control}$ effluents when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average ± standard deviation of two measurements (*n*=2).

SRT_{ext} (d)	**TrOC**	**Influent (ng/L)**	**Effluent (ng/L)**	
			SBR_{OSA}	**$SBR_{control}$**
10	Aspartame	63±6	145±11	207±27
	Paracetamol	79,000±12,728	125±16	278±144
	Triclosan	1,126±71	241±16	469±134
	4-tert-octylphenol	73±35	73±48	26±7
20	Atenolol	2,560±396	604±51	952±76
	Aspartame	20±0	48±19	229±287
	Salicylic acid	Below detection limit	874±419	406±167
	Caffeine	91,500±707	274±11	122±82
	Ketoprofen	107±4	35±11	Below detection limit
	Paracetamol	103,100±16,263	116±8	163±73
	Naproxen	4,650±240	509±7	187±13
	Ibuprofen	24,700±2,121	Below detection limit	77±0
	Estriol	291±25	Below detection limit	43±0
	Androstenedione	368±374	Below detection limit	17±0
	Bisphenol A	745±1	202±6	123±40
	Oxybenzone	161±20	66±3	Below detection limit
	Sulfamethoxazole	3,100±368	758±34	414±3
	Nonylphenol	20±1	13±0	44±36
40	Caffeine	19,500±283	63±46	563±26
	Ketoprofen	32±4	7±2	17±1
	Paracetamol	36,450±70	170±102	30±9
	Ibuprofen	5,525±247	23±16	240±3
	Gemfibrozil	232±4	23±15	138±17
	Estriol	175±0	Below detection limit	7±0
	Estrone	32±0	6±1	43±1
	Bisphenol A	1,020±1203	16±6	67±8
	Sulfamethoxazole	280±13	107±23	204±4
	Triclosan	428±5	226±131	158±19

OSA is not expected to achieve water of reuse standard, however, it is interesting note that the majority of the TrOCs in SBR_{OSA} and $SBR_{control}$ effluents met the Australian Guidelines for Water Recycling that was primarily based on drinking water standards and toxicological data (NRMMC/EPHC/NHMRC, 2008) (Table 8.3). A few compounds in both SBR_{OSA} and $SBR_{control}$ effluent exceeded the threshold concentrations, namely, caffeine, benzotriazole, estrone, and triclosan. Caffeine was highly biodegraded (Figure 8.2), but its influent concentration (19,500-91,500 ng/L, Figure 8.1) was very high possibly due to widespread human consumption (Luo *et al.*, 2014). Thus, although caffeine removal was high, a considerable amount of the contaminant remained in SBR_{OSA} and $SBR_{control}$ effluents. Caffeine has a relatively short half-life in estuarine waters (100 d), and its chronic impact on aquatic species is not yet fully understood (Moore *et al.*, 2008). The presence of high concentrations of caffeine in receiving waters implies significant anthropogenic impact on the environment (Moore *et al.*, 2008). Benzotriazole (an industrial anticorrosive) had low biodegradation and sorption on sludge (Figure 8.2). Therefore, its concentration in the influent (600-800 ng/L) was similar to that of the SBR_{OSA} and $SBR_{control}$ effluents (Figure 8.2a). This confirms previous findings showing that benzotriazole was poorly biodegraded in full-scale CAS (Janssen *et al.*, 2015). Benzotriazole is potentially genotoxic and carcinogenic (NRMMC/EPHC/NHMRC, 2008; Janssen *et al.*, 2015). Estrone has low biodegradation and tends to partition in the aqueous phase of sludge (Section 8.4.3.1). Estrone greatly impacts the growth behavior of aquatic plants and has endocrine disrupting effects in fish (Luo *et al.*, 2014). Triclosan had very low biodegradation (<20%), which is in agreement with literature (Kim *et al.*, 2014), and had significant sorption on sludge due to its high hydrophobicity (log D=5.15 at pH 7 and 25 ºC). Given that the influent triclosan concentration (428-1125 ng/L) was relatively high, residues were found in SBR_{OSA} and $SBR_{control}$ effluents. Triclosan has potential to have deleterious effect on nitrifying microorganisms in soil and inhibit the growth of specific flora (*e.g.*, cucumbers) (Waller and Kookana, 2009) .

Table 8.3. TrOC concentration in SBR_{OSA} and $SBR_{control}$ effluents in comparison with Australian samples and guidelines for water recycling. Caffeine, estrone, benzotriazole, and triclosan exceeded the recommended concentrations.

TrOC	Log D (pH 7; 25 ºC)	SBR_{OSA} effluent (ng/L)	$SBR_{control}$ effluent (ng/L)	Maximum reported for secondary effluents in Australia (ng/L) [a]	Australian guidelines (ng/L) [a]
TCEP	-5.19	148-806	145-777	540	1,000
Atenolol	-2.09	98-604	89-952	210	Not available
Aspartame	-1.99	48-145	207-229	1,700 (Phan *et al.*, 2015)	Not available
Salicyclic acid	-1.13	874	255-406	60,000	105,000
Saccharin	-1.09	161-165	138-148	340 (Trinh *et al.*, 2016)	Not available
Metoprolol	-0.81	103-132	100		Not available
[b] Caffeine	-0.63	63-379	122-564	44,000	350
Allopurinol	-0.55	-	6		Not available
Enalapril	-0.14	-	17-52	46	1,300
Ketoprofen	0.19	7-52	2180-2350	380	3,500
Sucralose	0.23	2027-2540	294-1101		Not available
Trimethoprim	0.27	256-1010	20-278	350	70,000
Paracetamol	0.47	116-170	187-330	4,300	175,000
Naproxen	0.73	090-316	11-91	570	220,000
Primidone	0.83	9-77	77-240	-	Not available
Ibuprofen	0.94	23	5-17	28,000	400,000
Triamterene	1.03	5-17	10	-	Not available
Fluoxetine	1.15	10	68-120	142	10,000
Dilantin	1.41	73-104	496-800	-	Not available
[b] Benzotriazole	1.42	536-770	496-800	2,400	7
Diclofenac	1.77	186-794	185-766	810	1,800
Phenylphenol	1.88	<5-29	<5-24	260	1×10^6
Carbamazepine	1.89	176-1184	163-112	27,000	100,000
Gemfibrozil	2.07	23-95	30	1,500	600,000
Verapamil	2.08	19-117	139	-	Not available

Hydroxyzine	2.15	-	71-121	-	Not available
Amtriptyline	2.28	37-347	48-245	-	Not available
Simazine	2.28	7	<5	1,000	20,000
Estriol	2.53	-	7-43	51	50
Diuron	2.68	13-135	14-127	290	30,000
Androstenedione	2.72	-	17	-	Not available
Diazepam	2.80	8	8	2.92×10^6	2.5×10^6
Propylparaben	2.88	-6-181177-23015-166	-	<5 (Trinh *et al.*, 2016)	Not available
[b] Estrone	3.13	7	43-116	110	30
Benzophenone	3.21	-	7-205	-	Not available
Clozapine	3.23	16-281	20-191	-	Not available
Levonorgestrel	3.37	-	5	-	Not available
Butylparaben	3.38	66-121	-	-	Not available
Bisphenol A	3.64	108-758	67-268	12,000	200,000
Diazinon	3.77	-	-	3,200	3,000
Oxybenzone	3.89	66-121	132		Not available
Sulfamethoxazole	3.90	108-758	204-414	1,900	35,000
Etiocholanolone	3.93	-	-	<5 (Phan *et al.*, 2015)	Not available
Androsterone	3.93	-	-	210	14,000
Ethynylestradiol	4.11	-	-	270	1.5
Testosterone	4.11	-	-	210	7,000
17a-estradiol	4.15	-	-	93	175
17b-estradiol	4.15	-	3	93	175
[b] Triclosan	5.15	226-525	160-469	400	350
t-Octylphenol	5.18	73-88	26-99	14	50,000
Nonylphenol	7.63	<5-13	<5-44	2,900	500,000
Triclocarban	12.80	87-316	109-439	50	Not available

[a] Obtained from the Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (NRMMC/EPHC/NHMRC, 2008) unless specified otherwise; [b] TrOCs that exceeded the threshold values.

The current findings suggest that although a contaminant is highly biodegraded or sorbed on sludge, the amount remaining in the effluent may still be of environmental concern. The concentrations of caffeine, benzotriazole, estrone, and triclosan in SBR_{OSA} and $SBR_{control}$ effluents were comparable to the maximum levels observed in secondary effluents across Australia (NRMMC/EPHC/NHMRC, 2008; Phan *et al.*, 2015; Trinh *et al.*, 2016). Further investigation on the environmental impact of these contaminants and an assessment of potential remediation technologies are highly recommended.

8.4.4 TrOC concentration in SBR sludge

To assess the effect of OSA on the occurrence of TrOCs in biosolids, the sorption of TrOCs on SBR_{OSA} sludge was determined. The concentrations of selected TrOCs in the solid phase of sludge are presented in Figure 8.2b. The sorption of TrOCs on sludge depended on electrostatic and hydrophobic interactions. Overall, SBR_{OSA} and $SBR_{control}$ had similar TrOC concentration in the solid phase of sludge. This indicates that OSA did not impact TrOC sorption in the SBR.

8.4.4.1 SBR_{OSA} sludge

Despite their hydrophilic nature, verapamil and amitriptyline (log D=2.08 and 2.28, respectively; pH7; 25 ºC) preferentially sorbed on sludge (Figure 8.2) possibly due to electrostatic interactions. These two compounds are positively-charged whereas the sludge surface is negatively-charged under neutral environment (Stevens-Garmon *et al.*, 2011). High sorption of verapamil and amitriptyline on sludge has been previously reported (Stevens-Garmon *et al.*, 2011). The current results indicate that electrostatic binding was an auxiliary sorption mechanism since other positively-charged but highly hydrophilic compounds (*e.g.*, atenolol, log D = -2.09; pH 7; 25 ºC) had low sorption. In other words, sorption through electrostatic interactions did not occur for TrOCs with high hydrophilicity.

Biodegradable hydrophilic TrOCs (log D<3) were rarely detected in SBR_{OSA} sludge with the exception of caffeine, paracetamol (Figure 8.2b), and ibuprofen (Figure A.7b). These three compounds had the highest concentration in domestic sewage (Section 8.4.2). Due to their high biodegradation, the concentration in the solid phase of sludge (<100 ng/g MLSS; $MLSS_{SBROSA}$=1-2 g/L) was much lower than that of the influent load (1,000-80,000 ng/L). The other hydrophilic compounds (*e.g.*, TCEP, sulfamethoxazole, dicloflenac, and others) that were

detected in SBR_{OSA} sludge were primarily non- or partially-biodegradable (Figure 8.2b and Figure A.7b). Because of their hydrophilic nature, a greater proportion of residual TrOCs was detected in the SBR_{OSA} effluent than sludge.

Among the hydrophobic TrOCs (log D>3), triclosan, triclocarban (Figure 8.2b), and clozapine (Figure A.7b) had the greatest concentration in the sludge solid phase of SBR_{OSA}. The concentration of the aforementioned compounds in sludge surpassed that of the influent load, indicating that previous loads sorbed and accumulated in sludge. All three compounds had EWGs (*e.g.*, –Cl) that potentially contributed to their low biodegradation. The positive charge of clozapine at neutral pH probably perpetuated its sorption (Stevens-Garmon *et al.*, 2011). Triclosan and triclocarban had the highest log D values among the TrOCs analysed in this study, and thus they sustained the highest concentration (>500 ng/kg) in SBR_{OSA} sludge (Figure 3b). With the exception of estrone (Section 8.4.3.1), residues of other and non- or partially-biodegradable hydrophobic TrOCs (*e.g.*, benzophenone, bisphenol A, and others) were more often found in SBR_{OSA} sludge than the effluent (Figure A.7b).

8.4.4.2 Comparison of SBR_{OSA} and $SBR_{control}$ sludge

Most TrOCs had nominal variation (less than 10%) in SBR_{OSA} and $SBR_{control}$ sludge at different SRT_{ext}, indicating that OSA did not affect the sorption of TrOCs in sludge. This was probably because the volume of sludge interchanged among the reactors and the change in reactor medium was relatively low, and thus dramatic change in the TrOC profile of sludge was not observed (Table 8.4)

Table 8.4. Flow rate and change in receiving media during sludge interchange in the OSA system when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average ± standard deviation of two measurements (*n*=2).

SRT_{ext} (d)	**q_1 (mL/d) [a] / Δ receiving media (%) [b]**	**q_2 (mL/d) [c] / Δ receiving media (%) [b]**	**q_3 [d] (mL/d)**	**q_4 [e] (mL/d) / Δ receiving media (%) [b]**	**q_5 (mL/d) [f] / Δ receiving media (%) [b]**
10	468 / 23.4	200/10	400	132/6.6	68/1.4%
20	234 / 11.7	100/5	200	66/3.3	34/0.7%
40	117 / 5.9%	50/2.5	100	33/1.7	17/0.4%

[a] q_1= SBR_{OSA} to aerobic/anoxic
[b] Δ receiving media (%) = volume transferred to the reactor/total volume of the reactor x 100
[c] q_2= aerobic/anoxic to anoxic
[d] q_3= wasted from aerobic/anoxic
[e] q_4= anoxic to aerobic/anoxic
[f] q_5= anoxic to SBR_{OSA}

Of the TrOCs that showed remarkable difference (more than 30%) between SBR_{OSA} and $SBR_{control}$ (Table 8.5), only caffeine, paracetamol, and ibuprofen were highly biodegraded in the SBRs (Section 8.4.3), and therefore the residual sludge concentration was negligible compared to the influent load (1,000-80,000 ng/L). The rest of the compounds were non- or partially biodegradable (*e.g.*, TCEP, benzophenone, and others) (Section 8.4.3), which explains why varying amounts were detected in the sludge solid phase.

Table 8.5. TrOCs with notable variation (more than 30% difference) in the solid phase of SBR_{OSA} and $SBR_{control}$ when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average of two measurements (n=2).

SRT_{ext} (d)	TrOC	SBR_{OSA} (ng/g dry solids)	$SBR_{control}$ (ng/g dry solids)
10	TCEP	45±5	68±4
	Atenolol	19±42	162±42
	Salicylic acid	Below detection limit	10,283±4,434
	Caffeine	97±16	217±7
	Diclofenac	150±9	244±2
	Amtriptylene	173±18	97±1
	Estrone	11±2	21±29
	Benzophenone	83±18	44±3
	Clozapine	141±14	88±15
	Bisphenol A	69±3	304±230
	Oxybenzone	119±13	53±11
	Sulfamethoxazole	100±24	49±14
20	TCEP	123±93	58±9
	Caffeine	107±19	178±22
	Sucralose	129±5	65±11
	Paracetamol	Below detection limit	159±2
	Ibuprofen	19±2	33±17
	Diclofenac	222±32	171±1
	Gemfibrozil	15±0	46±3
	Verapamil	169±4	238±10
	Amtriptylene	191±7	410±37
	Estrone	43±3	14±2
	Bisphenol A	1,922±2,620	111±1
	Oxybenzone	Below detection limit	85±4
	Sulfamethoxazole	268±37	Below detection limit
	Triclocarban	6,810±91	8,931±412
40	Atenolol	Below detection limit	30±1
	Estrone	5±1	22±1
	Clozapine	33±3	47±33
	Bisphenol A	17±5	38±11
	Triclocarban	2543±195	1,541±533

8.4.5 Impact of redox regimes in OSA external reactors

It is interesting to determine the fate of TrOC in the external reactors, which lends OSA additional redox regimes that make the system different from CAS. The aerobic/anoxic reactor, which received sludge from SBR_{OSA} and also from the anoxic reactor (Chapter 3, Figure 3.1a), had varying redox condition and was deficient in substrate. The anoxic reactor received sludge solely from the aerobic/anoxic reactor (Chapter 3, Figure 3.1a). It was continuously deficient in oxygen and substrate, and caused volatile solids destruction (Semblante *et al.*, 2016).

8.4.5.1 Aerobic/anoxic reactor

To determine the fate of TrOCs in the external aerobic/anoxic reactor, its aqueous and solid phase TrOC concentrations were compared with that of SBR_{OSA} and anoxic reactor at each SRT (Figure A.8). The concentrations of selected TrOCs are presented in **Figure 8.3**. To assess TrOC sorption and biodegradation, the concentration of individual TrOCs entering the aerobic/anoxic reactor ($Y_{in\text{-}ae/anx}$) was estimated (Section 8.3.4.2) and compared with the actual concentrations detected in the reactor (Figure 8.4).

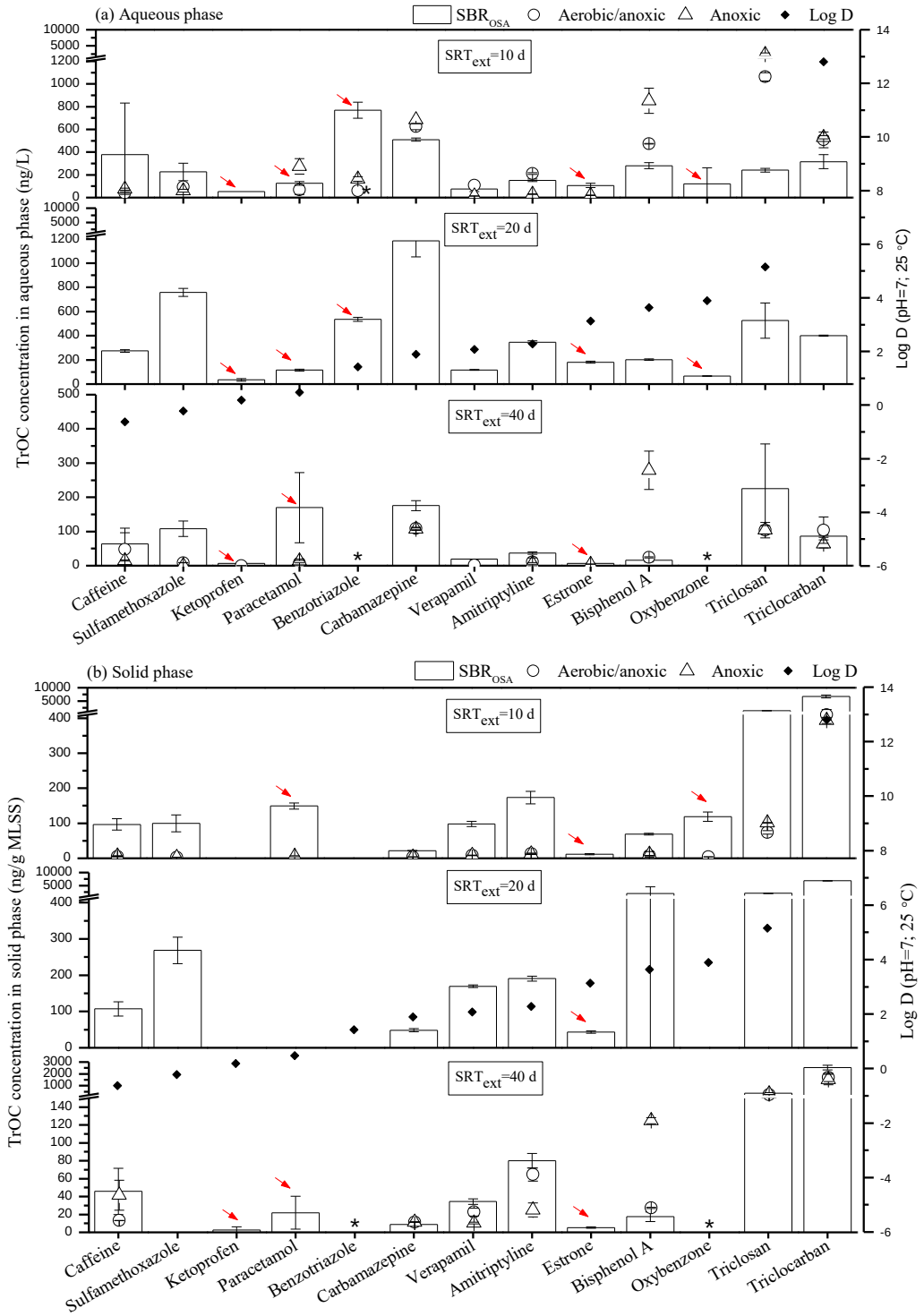

Figure 8.3. Concentration of selected TrOCs in the (a) aqueous and (b) solid phases of the external aerobic/anoxic and anoxic reactor of OSA when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average of two measurements (n=2). The asterisks (*) represent contaminants that were not analysed in a particular sampling campaign. The arrows (→) denote contaminants that were highly biodegraded in the aerobic/anoxic reactor. Only estrone was highly biodegraded in the anoxic reactor.

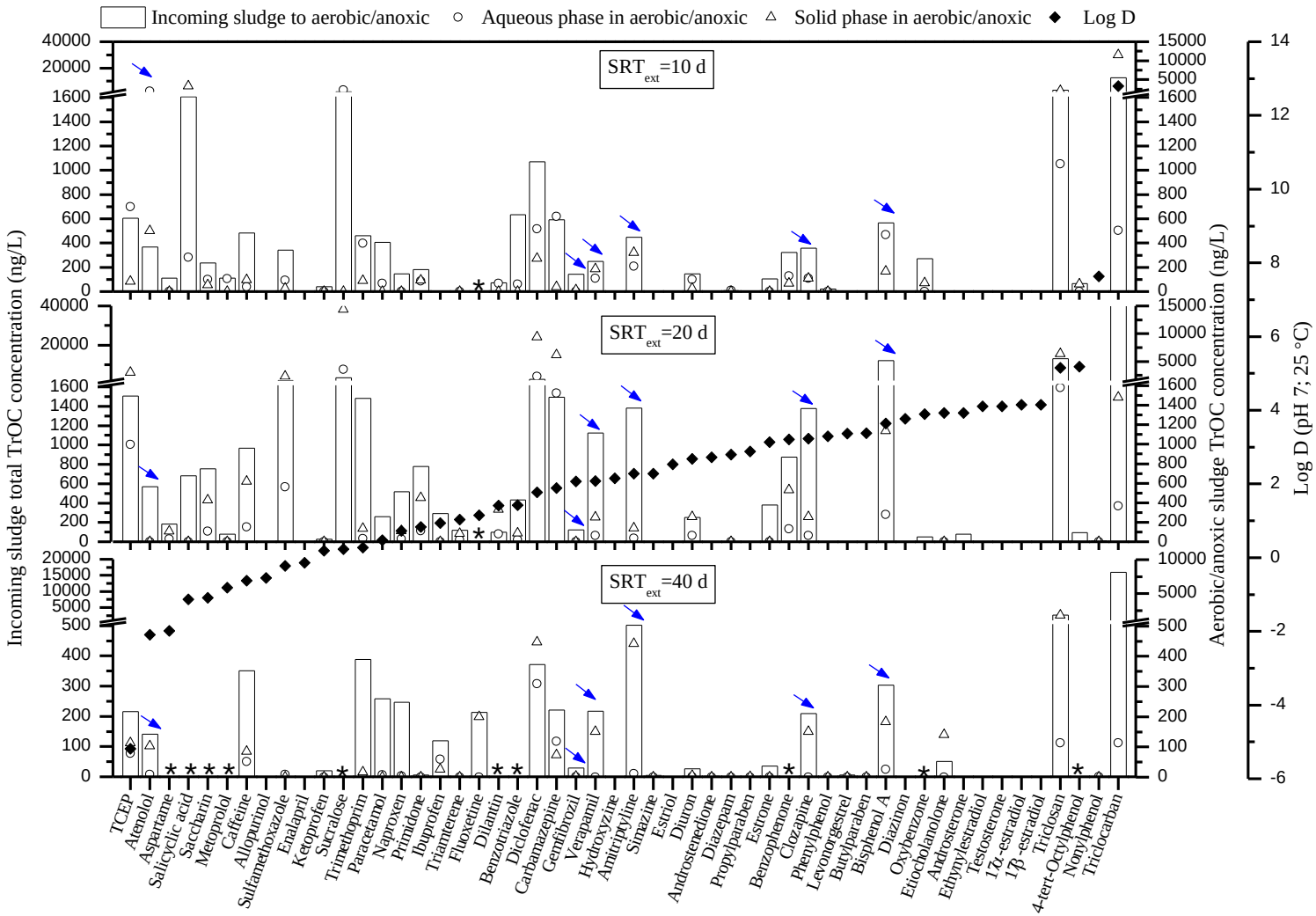

Figure 8.4. The concentration TrOCs entering the aerobic/anoxic reactor ($Y_{in\text{-}aerobic/anoxic}$, labelled as "incoming sludge") *vs.* the concentration of TrOCs in aqueous and solid phase of sludge in aerobic/anoxic reactor when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average of two measurements. The asterisks (*) represent contaminants that were not analysed in a particular sampling campaign. The biodegradation of some TrOCs (denoted by arrows →) increased when SRT_{ext} was increased from 10 to 20 d, but decreased when SRT_{ext} was further increased to 40 d.

Results show that ketoprofen, paracetamol, estrone, oxybenzone (**Figure 8.3**), naproxen, and gemfibrozil (Figure A.8) were highly biodegraded in the aerobic/anoxic reactor. The aqueous and sludge phase concentrations of these TrOCs in the aerobic/anoxic reactor were significantly lower than that of the incoming sludge (Figure 8.4). Additionally, benzotriazole (which was not analysed at SRT_{ext} of 40 d) was highly biodegraded in the aerobic/anoxic reactor when SRT_{ext} was 10 and 20 d (**Figure 8.3**). Among the aforementioned compounds, ketoprofen, paracetamol, and naproxen were easily biodegraded in SBR_{OSA} (Section 3.2.1), and therefore the load to the external reactors was relatively low (10-250 ng/L) in comparison with non- or partially-biodegradable TrOCs. Gemfibrozil was partially biodegraded in SBR_{OSA} despite having only EDGs (*e.g.*, methyl and ether groups) (Table B.3) attached to the aromatic ring probably because of the low SRT of SBR_{OSA} (10 d) (Section 3.2.1).

Benzotriazole, estrone, and oxybenzone were poorly biodegraded in SBR_{OSA} (Section 8.4.3.1), but they were highly biodegraded in the aerobic/anoxic reactor (**Figure 8.3**). Benzotriazole was probably biodegraded in the aerobic phase. In essence, the aerobic/anoxic reactor provided extended aeration that consequently enhanced TrOC removal in recirculated sludge. Previous research using batch experiments showed that benzotriazole was biodegraded in aerobic but not in either anoxic or anaerobic conditions (Herzog *et al.*, 2014). Previous reports showed that the biodegradation of pharmaceuticals by heterotrophic bacteria increased when aerobic treatment was increased (Clara *et al.*, 2005; Huang *et al.*, 2008). It has been observed that 40-50 d of incubation was necessary to achieve near complete removal of benzotriazole by aerobic treatment (Herzog *et al.*, 2014). This is the first study showing improvement in benzotriazole biodegradation through the addition of external reactors in the return activated sludge loop.

Meanwhile, the biodegradation of estrone and oxybenzone probably occurred during the anoxic phase. Under anoxic conditions, the ketone group of estrone is reduced to a hydroxyl group to form 17β-estradiol (Shi *et al.*, 2013). Since 17β-estradiol was not detected in either aqueous or solid phase of the aerobic/anoxic reactor, further biodegradation in either aerobic or anoxic phases could be inferred. There is limited information on the biodegradation pathways of oxybenzone in sludge. Nonetheless, the results of this study is corroborated by Phan *et al.* (2014), who reported that the oxybenzone removal of MBR was enhanced by internal aerobic-

anoxic recirculation. This suggests that the biodegradation of oxybenzone was possibly due to the action of denitrifying microorganisms (during the anoxic phase).

The majority of the TrOCs had varying but generally poor biodegradation rates in the aerobic/anoxic reactor at different SRT_{ext}, which suggests that they were non-biodegradable (Figure A.8 and Figure 8.4). Poor biodegradation at substrate-deficient conditions suggests that co-metabolism is the primary mechanism involved in TrOC biotransformation. In other words, many TrOCs are incapable of standing as primary carbon source for microbial maintenance. Instead, these TrOCs are catabolised only when other carbon sources are available (Semblante *et al.*, 2015a). Due to substrate deficiency in the external reactors, further biodegradation of these compounds will not occur unless sludge is recirculated back to the aeration tank.

Some TrOCs, such as verapamil and amitriptyline (**Figure 8.3**), were poorly biodegraded and thus accumulated in aerobic/anoxic sludge. Because sludge is wasted from the aerobic/anoxic reactor in this particular configuration (Chapter 3, Figure 3.1a), the aforementioned compounds represent the typical TrOC emission profile of OSA. There was a lower variety of TrOCs in the aerobic/anoxic reactor sludge compared to SBR_{OSA} sludge (**Figure 8.3**) due to the biodegradation of some compounds (*e.g.*, estrone and benzotriazole) in the former reactor. However, the concentration of recalcitrant and sorbing TrOCs was higher in the aerobic/anoxic reactor compared to SBR_{OSA} (**Figure 8.3**). For instance, the concentrations of triclosan (266-1,477 ng/g MLSS) and triclocarban (1,886-8,384 ng/g MLSS) in the aerobic/anoxic reactor were three and sixteen times greater than in SBR_{OSA}/$SBR_{control}$. The implications of these findings on the TrOC emission of OSA are discussed in Section 8.4.8.

8.4.5.2 Anoxic reactor

To determine the fate TrOCs in the anoxic reactor, the TrOC concentration in the aqueous and solid phases of the anoxic and aerobic/anoxic reactors was compared (Figure A.8). Additionally, the total concentration of TrOCs entering the anoxic reactor ($Y_{in\text{-}anoxic}$) was estimated (Section 8.3.4.2) and compared with the actual concentrations in the reactor to gain further insight on sorption and biodegradation (Figure 8.5).

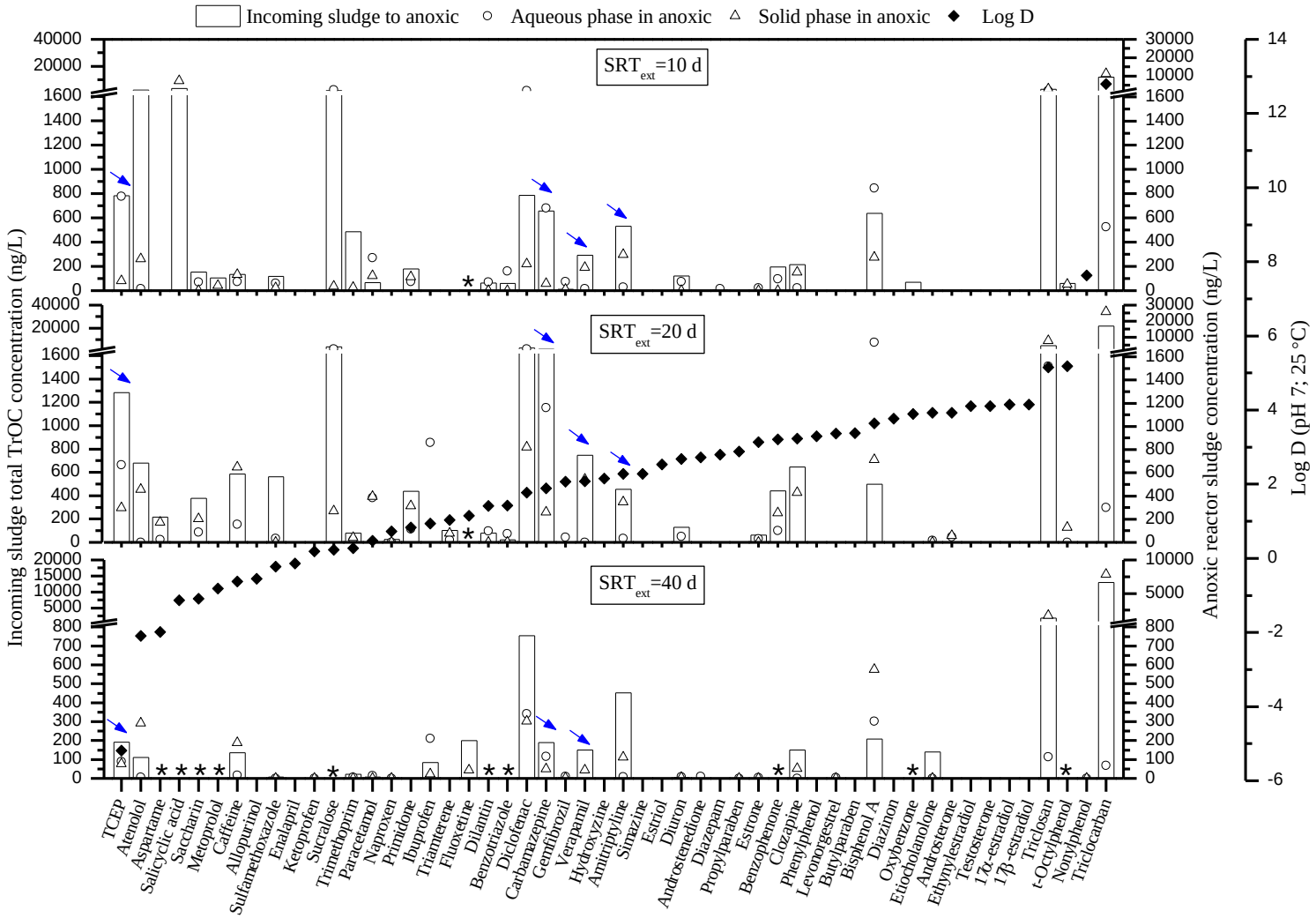

Figure 8.5. The concentration TrOCs entering anoxic reactor ($Y_{in\text{-}anoxic}$, labelled as "incoming sludge") *vs.* the concentration of TrOCs in aqueous and solid phase of sludge in anoxic reactor when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average of two measurements. The asterisks (*) represent contaminants that were not analysed in a particular sampling campaign. The biodegradation of some contaminants (denoted by arrows →) increased when SRT_{ext} was increased from 10 to 40 d.

Generally, there was poor biodegradation of TrOCs in the anoxic reactor relative to the aerobic/anoxic reactor or SBR_{OSA}. A few TrOCs such verapamil, amitriptyline, carbamazepine (**Figure 8.3**), TCEP, and clozapine (Figure A.8) had some biodegradation (*e.g.*, 20-30%) especially when SRT was increased from 10 to 40 d (to be discussed in Section 8.4.6). The rest of the TrOCs were recalcitrant under anoxic treatment.

Interestingly, the aqueous phase concentration of some TrOCs in the anoxic reactor was greater than that of the aerobic/anoxic reactor and the incoming sludge. These included paracetamol, carbamazepine, bisphenol A, triclosan (**Figure 8.3**) sucralose, ibuprofen, and diclofenac (Figure A.8). A closer inspection of the data showed that the aforementioned compounds originally partitioned in the solid phase of the aerobic/anoxic reactor, but were possibly released in aqueous phase of the anoxic reactor. Previous research demonstrated that the key sludge reduction mechanism of OSA is sludge autolysis in the anoxic reactor (Semblante *et al.*, 2016). The destruction of solids probably resulted in the loss of TrOC sorption sites which led to the desorption of contaminants that were sorbed on sludge. The desorption of TrOCs, such as estrogens and nonylphenol, as a direct result of solids destruction during biological or advanced oxidation treatment has been reported in literature (Chawla *et al.*, 2014; Semblante *et al.*, 2015b). Nonetheless, this is the first report confirming desorption of TrOCs (specifically pharmaceuticals, artificial sweeteners, and industrial chemicals) from sludge during application of a biological sludge reduction strategy. Notably, in this particular OSA configuration, sludge is discharged from the aerobic/anoxic rather than the anoxic reactor (Chapter 3, Figure 3.1a) where TrOC desorption occurs. Therefore, this configuration helps minimise the emission of TrOCs in the aqueous phase.

8.4.6 Impact of SRT_{ext} on TrOC biodegradation in external reactors

The biodegradation of certain TrOCs exhibited dependence on SRT_{ext}. In the aerobic/anoxic reactor, some TrOCs (*e.g.*, caffeine and primidone) had the highest biodegradation at SRT_{ext} of 40 d while others (*e.g.*, verapamil and bisphenol A) had the highest at 20 d (the optimum condition for sludge reduction). Meanwhile, in the anoxic reactor, increasing SRT_{ext} from 10 to 40 d slightly increased the biodegradation of a few TrOCs (*e.g.*, TCEP and clozapine). Although changing SRT_{ext} varied the biodegradation rates of certain TrOCs, it did not result in complete biodegradation of any contaminant in either aerobic/anoxic or anoxic reactor.

The biodegradation of caffeine (**Figure 8.3**) and primidone (Figure A.8) in the aerobic/anoxic reactor was enhanced when SRT_{ext} was increased from 10 to 40 d although a complete biodegradation of either compound was not observed. Previously, the cleavage of the imidazole ring of caffeine was observed in anoxic sediments (Bradley *et al.*, 2007). Meanwhile, primidone was biodegraded well by aerobic-anoxic sludge interchange in an MBR (Phan *et al.*, 2014). Therefore, the improvement in biodegradation of these two compounds at SRT_{ext} of 40 d could be attributed to longer reaction time under anoxic condition.

The biodegradation of some compounds such as verapamil, amitriptyline, bisphenol A (**Figure 8.3**), atenolol, gemfibrozil, and clozapine (Figure 8.4) in the aerobic/anoxic reactor slightly increased when SRT_{ext} was increased from 10 to 20 d, but decreased when SRT_{ext} was 40 d. Our previous study showed that SRT_{ext} of 20 d favoured nitrification/denitrification in the aerobic/anoxic reactor and helped facilitate the cycle of sludge autolysis in OSA (Semblante *et al.*, 2016). The high biodegradation of the aforementioned compounds at this condition might be attributed to the activity of nitrifiers and denitrifiers. Indeed, denitrifiers had the highest abundance in the aerobic/anoxic reactor at SRT_{ext} of 20 d (Chapter 7, Section 7.4.4.3). A linkage between TrOC biodegradation and nitrification/denitrification has been reported by other researchers. For example, Phan et al. (2014) reported that the anoxic condition was responsible for the removal (50-90%) of amitriptyline in an MBR. Tran et al. (2014) demonstrated the linear relationship of nitrification and co-metabolic biodegradation of artificial sweeteners. However, unlike the aerobic/anoxic reactor in the current study, the reactors of Phan *et al.* (2014) and Tran *et al.* (2014) were not deficient in substrate. The lack of substrate in the aerobic/anoxic reactor explains why TrOC biodegradation was generally poor and further emphasises the relevance of co-metabolic pathways in TrOC biodegradation. The results of this study further suggest that optimizing nitrification/denitrification in the external reactors of OSA can synergistically facilitate sludge reduction (via the conversion of destroyed solids into inert products) and biodegradation of certain TrOCs.

A few TrOCs exhibited a slight increase in biodegradation in the anoxic reactor with increasing SRT (*e.g.*, TCEP, verapamil, amitriptyline, carbamazepine, and clozapine) although high biodegradation was not achieved (Figure 8.5). The improvement in verapamil and amitriptyline biodegradation in the aerobic/anoxic reactor was closely associated with nitrifying/denitrifying

efficiency (Section 8.4.5.1). Further biodegradation of verapamil and amitriptyline in the anoxic reactor shows that anoxic treatment was indeed conducive to their biodegradation. Unlike this study, high biodegradation of amitriptyline, carbamazepine, and clozapine has been reported in an anaerobic MBR (ORP=-200 mV) which was not deficient in substrate and had high methanogenic activity (Wijekoon *et al.*, 2015). In this study, the ORP of the anoxic reactor (-450 mV) was low but methanogenic activity (indicated by biogas production) was not observed probably because of substrate deficiency. Although a relationship between biodegradation and SRT was observed for the aforementioned compounds, the majority of the load from the incoming sludge was not biodegraded probably because co-metabolic degradation pathways were not activated in the absence of substrate. The residues partitioned in varying concentrations in the aqueous and/or solid phase of anoxic sludge (Figure 8.5).

8.4.7 $SBR_{control}$ vs. aerobic digester: Impact of substrate deficiency

Aerobic digestion involves the treatment of sludge in a completely mixed aerated reactor. The fate of TrOCs in the aerobic digester was investigated to assess the TrOC emission of a conventional sludge treatment unit (Figure A.9). The concentrations of selected TrOCs are presented in Figure 8.6. The total concentration of TrOCs entering the aerobic digester ($Y_{in\text{-}aerobic}$) was estimated (Section 8.3.4.2) and compared with the actual concentrations in the reactor to gain further insight on sorption and biodegradation (Figure 8.7). Furthermore, the biodegradation of TrOCs in $SBR_{control}$ (Figure A.7) was compared with that of the aerobic digester to determine the impact of substrate deficiency in TrOC removal (Figure A.9).

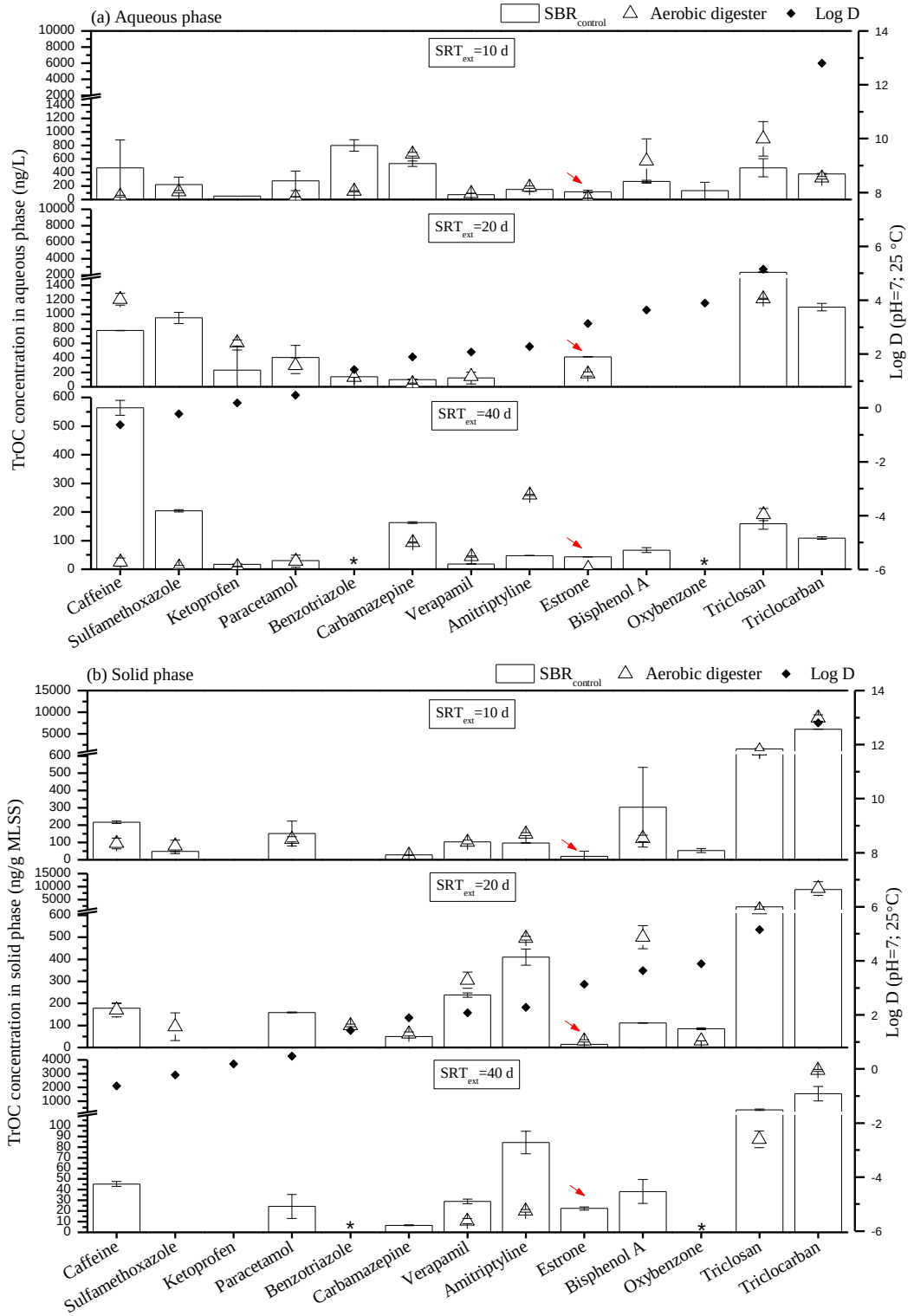

Figure 8.6. Concentration of selected TrOCs the (a) aqueous and (b) solid phase of sludge in the external control aerobic digester when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average of two measurements (*n*=2). The asterisks (*) represent contaminants that were not analysed in a particular sampling campaign. The arrows (→) denote contaminants that were highly biodegraded in the aerobic digester (estrone only).

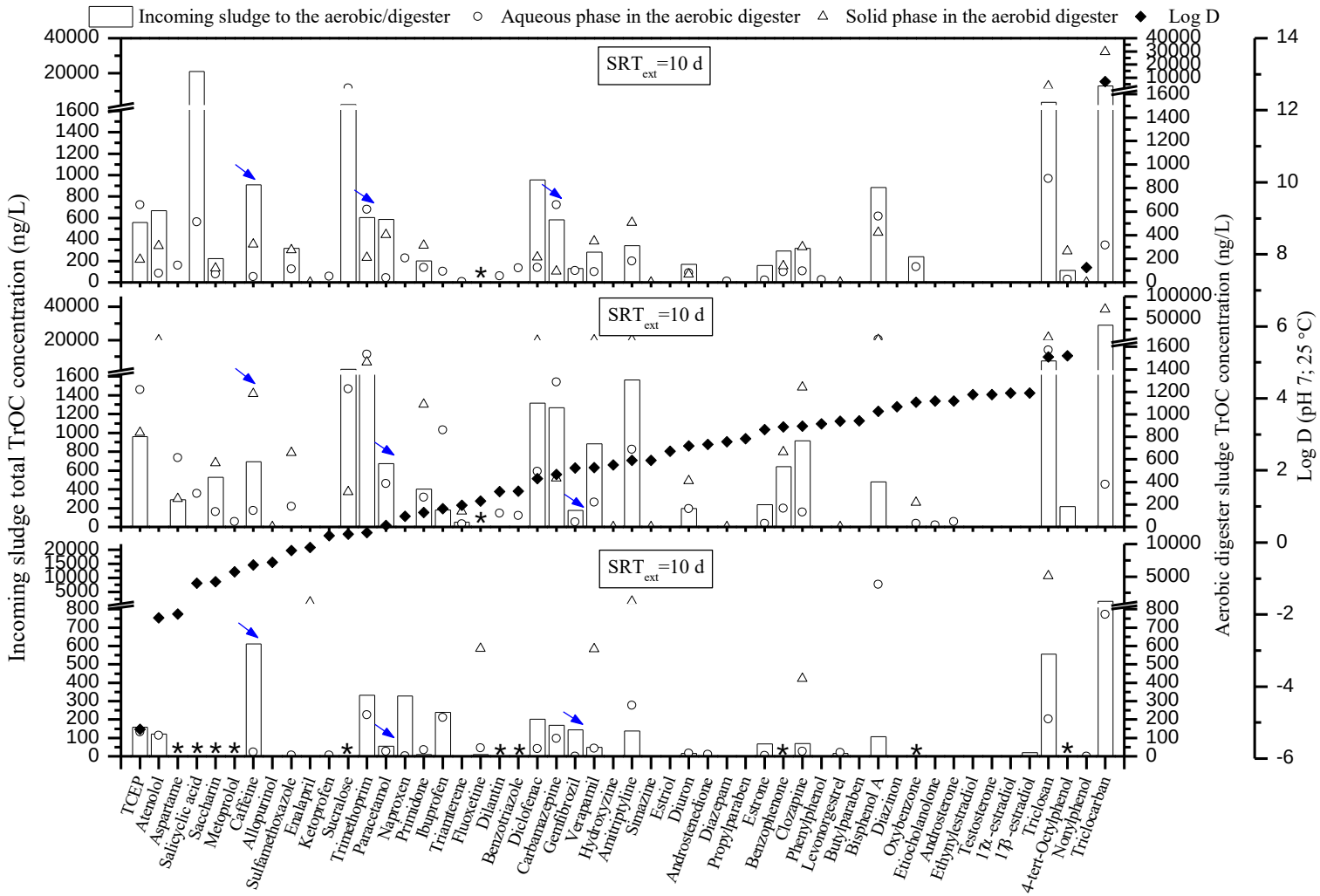

Figure 8.7. The concentration TrOCs entering control aerobic digester ($Y_{in\text{-}\ aerobic}$, labelled as "incoming sludge") *vs.* the concentration of TrOCs in aqueous and solid phase of sludge in the aerobic digester when SRT_{ext} was varied (10-40 d) and SRT_{SBR} was maintained at 10 d. The values are the average of two measurements. The asterisks (*) represent contaminants that were not analysed in a particular sampling campaign.The biodegradation of some TrOCs (denoted by arrows →) increased when SRT_{ext} was increased from 10 to 40 d.

$SBR_{control}$ and aerobic digester were both under aerobic condition, but the former was fed with influent (domestic sewage) with relatively high concentration of TrOCs and the latter was fed with sludge containing low concentration of readily biodegradable sCOD and reduced concentration of TrOCs. In other words, $SBR_{control}$ and aerobic digester were rich and deficient in substrate, respectively. A comparison of the fate of TrOCs in the two reactors provides useful insight on the role of substrate availability on TrOC biodegradation. Generally, with a few exceptions (Section 8.4.3), treatment in either $SBR_{control}$ or SBR_{OSA} resulted in (i) up to 80% biodegradation of hydrophilic TrOCs especially those with EDG and, (ii) poor biodegradation of hydrophobic TrOCs especially those with EWG. On the contrary, only estrone (a hydrophobic TrOC that was poorly biodegraded in $SBR_{control}$, Section 8.4.3) was consistently biodegraded at different SRT_{ext} in the aerobic digester (Figure 8.6). Additionally, a few TrOCs (*e.g.*, caffeine, naproxen, and gemfibrozil) were highly biodegraded in the aerobic digester only at SRT_{ext} of 40 d (Figure A.9 and Figure 8.7). This demonstrates that the biodegradation of many TrOCs under aerobic condition occurs only when primary substrate is available. This further shows that many TrOCs are aerobically biodegraded by participating as secondary substrate in co-metabolic pathways (Semblante *et al.*, 2015b).

8.4.8 Insights on the TrOC emission from OSA

TrOC emission from the particular OSA configuration used in this study was assessed by comparing TrOC concentrations in SBR_{OSA}, the aerobic/anoxic reactor (where sludge is discharged from the OSA system; Chapter 3, Figure 3.1a), and the control aerobic digester (where sludge is discharged from the control system; Chapter 3, Figure 3.1b). The aerobic/anoxic reactor (**Figure 8.3**) generally showed lower concentration of many TrOCs in both aqueous and solid phases than SBR_{OSA} (**Figure 8.3**) given that the majority of the contaminants have already been biodegraded in the main aeration tank.

The aerobic/anoxic reactor also enhanced the biodegradation of estrone, oxybenzone, and benzotriazole (Section 8.4.5.1). However, non-biodegradable TrOCs (*e.g.*, triclosan and triclocarban) accumulated in the aerobic/anoxic reactor and therefore the solid phase concentration was higher than that of SBR_{OSA} (Section 8.4.5.2). In other words, treatment of sludge in the external reactors of OSA enhanced the biodegradation of some TrOCs (*e.g.*, benzotriazole, **Figure 8.3**a) but resulted in the accumulation of others (*e.g.*, triclosan, **Figure**

8.3b) especially those that are hydrophobic and non-biodegradable in either aerobic or anoxic condition.

Notably, this particular OSA configuration discharges sludge from an aerobic/anoxic reactor rather than an anoxic reactor, which is commonly found in literature (Goel and Noguera, 2006; Semblante *et al.*, 2014). The current study revealed that the aerobic/anoxic treatment resulted in greater biodegradation of TrOCs than the anoxic treatment (Section8.4.5). Moreover, the destruction of volatile solids in the anoxic reactor caused desorption of some TrOCs (*e.g.* paracetamol, sucralose, and bisphenol A) from the solid phase of sludge and consequently increased TrOC concentration in the aqueous phase (Section 3.5.2). This is an indication that the current OSA configuration has potential to have lower TrOC emission than others involving a single external anoxic reactor.

It is interesting to compare the TrOC concentration in the final residues of OSA and control systems (Chapter 3, Figure 3.1). Generally, the aerobic/anoxic and anoxic reactors of OSA resulted in the biodegradation of a greater number of TrOCs than the aerobic digester. The superior performance of the aerobic/anoxic reactor can be attributed to the variation in redox conditions, which gave rise to nitrifying/denitrifying bacteria that potentially facilitated the biodegradation of some recalcitrant TrOCs (Section 3.5). Highly hydrophobic TrOCs such as triclosan and triclocarban were the most persistent contaminants in biosolids. The concentration of triclosan and triclocarban in the aerobic digester (406-10,413 ng/g MLSS) was higher than that of the aerobic/anoxic reactor of OSA (266-8,384 ng/g MLSS). This shows that OSA has potential to yield higher quality biosolids compared to aerobic digestion. To further enhance TrOC biodegradation in OSA, it will be worthwhile to perform further study at longer SRT_{ext} (>40 d). This may result in greater diversification of bacteria and metabolic pathways or in longer reaction time. However, as demonstrated in Chapter 7, operating at SRT_{ext}>20 d do not result in further improvement in sludge reduction.

8.5 CONCLUSIONS

OSA did not affect the effluent TrOC concentration of the SBR. However, the biodegradation of estrone, benzotriazole, and benzophenone was enhanced in the aerobic/anoxic reactor. Generally, aerobic/anoxic favoured TrOC biodegradation than anoxic condition. Some TrOCs underwent

desorption from sludge due to volatile solids destruction under anoxic condition. The concentration of highly sorbing and recalcitrant TrOCs (*e.g.*, triclosan) in the aerobic/anoxic reactor was lower than that of the control aerobic digester. This suggests that the final sludge residue generated by OSA have potential to have lower TrOC content than that of CAS paired with aerobic digestion.

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CHAPTER 9: Conclusions and Recommendations

9.1 CONCLUSIONS

This study operated a laboratory-scale OSA system fed with domestic wastewater *(i.e.*, real wastewater) and determined the impact of three critical factors (addition of iron salt, SIR, and SRT) on sludge reduction. Results showed that depending on the operation conditions, OSA can reduce sludge yield by more than 35%. Moreover, by optimising OSA performance according to the aforementioned factors, the underlying mechanisms governing sludge reduction were elucidated. Essentially, sludge reduction was triggered by the interchange of sludge between conditions that are rich (the main aeration tank) and deficient (the external reactors) in oxygen and substrate. The external anoxic reactor was responsible for sludge autolysis, while the intermittently aerated (*i.e.*, aerobic/anoxic) reactor enabled nitrification/denitrification reactions that ensured lysates (products of cell lysis) were converted to inert forms. These reactions were driven by distinct shifts in microbial community structure of sludge under environmental stress. Additionally, OSA decreases the sludge yield of the main aeration tank by facilitating the growth of slow-growing bacteria (*e.g.*, nitrifiers). Furthermore, for the first time, this study revealed the fate of TrOCs in OSA and demonstrated that OSA has potential to reduce TrOC concentration in residual biosolids.

In Chapter 2, a comprehensive review of OSA and other technologies that cycle sludge in different redox regimes was performed. The potential mechanisms of sludge reduction and the factors affecting them were critically analysed. The potential impact of OSA on wastewater treatment efficiency and sludge properties were discussed. Moreover, to gain insight on the potential fate of TrOCs in OSA, the sorption and biodegradation of TrOCs in the wastewater and sludge treatment lines were evaluated. Relevant information was gleaned on biotransformation pathways under different redox conditions and operation conditions.

Chapter 4 discusses how $FeCl_2$ addition affects sludge reduction in alternating redox conditions. Fe(II) was oxidised to Fe(III) under aerobic condition. The first part of the study, a batch experiment was performed involving four batch reactors (aerobic/anoxic, aerobic/anoxic+$FeCl_2$, anoxic, anoxic+$FeCl_2$). Results showed that adding 30 mg/L of $FeCl_2$ decreased the volatile solids reduction under aerobic/anoxic conditions probably due to a decline in the destructibility of EPS. The second part of the study, a continuous experiment was performed involving the

operation of laboratory-scale OSA and control systems fed with settled domestic sewage dosed with varying $FeCl_2$ concentration (none, 15, and 30 mg/L) to the influent. The results of the continuous experiment corroborated that of the batch experiment, showing that adding at least 15 mg/L of $FeCl_2$ to the influent increased the EPS concentration of the aerobic/anoxic reactor. Without $FeCl_2$ addition, the sludge yield (g sludge produced/g substrate consumed) of the continuously operated SBR_{OSA} was 24.8% lower than that of the $SBR_{control}$. Results suggested that without $FeCl_2$ addition of more than 15 mg/L, OSA reduced sludge in two ways: (i) it destroyed volatile solids of in the external reactors, and (ii) it decreased volatile solids production in the main aeration tank (*i.e.*, SBR_{OSA}).

Chapter 5 details how SIR affects sludge reduction in OSA when settled and unsettled domestic sewage was used as the influent. SIR is the percentage by volume of sludge returned from the external reactor to the main aeration tank of OSA. In the first part of the study, settled sewage was fed to the laboratory-scale OSA and control systems and the SIR of OSA was varied (11, 16.5, and 22%). An SIR of 11% increased sludge residence time in the external reactors and maximised sludge yield reduction. In the second part of the study, unsettled sewage was fed to the laboratory-scale systems and OSA was operated without and with SIR of 11%. Unsettled sewage represented wastewater with greater "strength" in terms of sCOD in comparison with settled sewage. Results showed that sludge reduction mechanisms were turned off in the absence of sludge interchange, and further confirmed that 11% was the optimum SIR for OSA operation. Higher influent strength resulted in greater volatile solids content in sludge fed into the external reactors of OSA, which enhanced volatile solids destruction under stressful conditions. Moreover, the study showed that an intermediate SIR (11%) increased sludge residence time in the external reactors and maximised OSA performance via two mechanisms: (a) providing optimum environment for volatile solids destruction as evidenced by the increase in orthophosphate under anoxic conditions, and (b) facilitating the conversion of lysed materials into inert forms as evidenced by the decrease in ammonia and nitrate under aerobic/anoxic conditions.

Chapter 6 details how SRT_{ext} impacts sludge reduction in OSA using unsettled sewage as the influent. The SRT_{ext} of the OSA and control systems were varied (10, 20, and 40 d). Results

showed that under the optimum SRT_{ext} of 20 d, OSA facilitated volatile solids destruction in the external anoxic reactor and nitrification/denitrification in the external aerobic/anoxic reactor. Increasing SRT_{ext} enhanced the autolysis of sludge under oxygen- and substrate-deficient conditions. However, beyond the optimum SRT_{ext} (20 d), further sludge reduction did not occur. Instead, a decrease in nitrification/denitrification efficiency in the external aerobic/anoxic reactor and consequently deteriorated OSA performance was observed. Furthermore, this study showed that aerobic/anoxic sludge interchange helps increase the dewatered cake solids content and reduce the CST of unconditioned sludge when an optimum SRT_{ext} was applied.

In Chapter 7, the microbial community structure of OSA at different SRT_{ext} is discussed. SBR_{OSA} had greater microbial diversity and contained more slow-growing nitrifying bacteria than $SBR_{control}$, which possibly explains why the former reactor had lower sludge yield than the latter. Constrained PCoA of unweighted Unifrac distances demonstrated that redox condition was the most important factor affecting microbial diversity. Generally, microbial diversity increased in the following order: aerobic<intermittent aerobic/anoxic<anoxic. Members of the class *β*- and *γ*-Proteobacteria decayed in the external reactors of OSA, suggesting that they did not survive under environmental stress (*i.e.*, oxygen- and substrate-deficient conditions). However, hydrolysing, fermentative, nitrifying, denitrifying, and predatory bacteria proliferated in the external reactors despite of environmental stress. Sludge autolysis in the external reactors was enhanced at SRT_{ext} of 20 d. Under this condition, the population of denitrifying (*e.g.*, order *Xanthomondales*) and predatory bacteria (*e.g.*, order *Myxobacteriales* and genus *Bdellovibrio*) increased in the external aerobic/anoxic and anoxic reactor, respectively. The mechanism of sludge reduction from a microbiological perspective is as follows: bacteria such as *β*- and *γ*-*Proteobacteria* decay in the external reactor, thereby producing materials that can be metabolised by bacteria that are enriched in under environmental stress (*e.g.*, hydrolyzers, fermenters, predators, nitrifiers, and denitrifiers). Results suggest that predators and denitrifiers played key roles in sludge autolysis and converting lysates into inert forms, respectively.

In Chapter 8, the fate of a wide range of TrOCs in OSA and control systems at different SRT_{ext} is discussed. SBR_{OSA} and $SBR_{control}$ had comparable effluent TrOC concentration, indicating that OSA did not affect TrOC biodegradation during aerobic treatment. The external aerobic/anoxic

reactor of OSA showed capacity to enhance the biodegradation of some compounds, such as estrone, benzotriazole, and benzophenone, possibly to unique biodegradation pathways occurring under alternating redox regimes. Generally, aerobic/anoxic condition favoured TrOC biodegradation than anoxic condition regardless of SRT_{ext}. Some TrOCs underwent desorption from sludge due to volatile solids destruction under anoxic condition, thereby increasing the aqueous phase TrOC concentration. This suggests that the current OSA configuration (involving external aerobic/anoxic and anoxic reactors) possibly have lower TrOC emission than others involving only an external anoxic reactor. Moreover, the concentration of highly sorbing and recalcitrant TrOCs (*e.g.*, triclosan and triclocarban) in the aerobic/anoxic reactor was lower than that of the aerobic digester. This indicates the final sludge residue of OSA has lower TrOC content than that of CAS paired with aerobic digestion.

9.2 RECOMMENDATIONS FOR FURTHER INVESTIGATION

Several research directions can be pursued to further enrich the state of the art of OSA and/or to address the issues regarding excess sludge production and poor effluent quality in WWTPs. First, the findings of this study should be verified in a pilot-scale OSA. Although real wastewater was used in the laboratory-scale continuous reactors, under- or over-estimation of sludge reduction could have occurred when influent strength was relatively low (due to wet weather) and sludge production rates of the SBRs intermittently varied. In Chapter 5 (Section 5.4.2.2) , it was observed that sludge reduction in OSA was more apparent when the influent strength and the volatile solids concentration of sludge loaded to the external reactors were relatively high. The potential effect of low influent strength can be overcome in a pilot-scale system possessing greater amount of biomass that can sustain high sludge production rates despite of intermittent changes in influent characteristics for short periods of time. Furthermore, results in pilot-scale studies can facilitate the transfer of OSA technology in full-scale plants.

Second, strategies should be developed to remove phosphorous from the effluent of OSA. This study showed that adding iron salts to the influent to remove phosphorous by chemical approach prevented sludge biodegradation in OSA (Chapter 4). To avoid the environmental impact of phosphorous (*e.g.*, eutrophication of receiving waters), phosphorous in the effluent can be treated using physical or chemical methods. An alternative approach is to recover phosphorous from the

effluent. To achieve this, additional technologies must be integrated in the wastewater treatment line to increase the concentration (*e.g.*, membrane filtration) and to retrieve (*e.g.*, adsorption or crystallization) phosphorous. The costs associated with these technologies could be offset by savings in phosphorous levies and/or revenue generated from phosphorous recovery.

Third, the effect of other relevant factors on sludge reduction in OSA can be investigated. External reactor temperature emerges as a factor that may have significant impact on volatile solids destruction. OSA and similar processes have thus far only been operated under ambient temperature (Chapter 2). However, studies have shown that increasing temperature improves floc destruction in thermophilic aerobic and anaerobic digesters due to kinetic acceleration of biochemical reactions and selection of thermophilic bacteria that could induce enzymatic hydrolysis of cell walls (Calace *et al.*, 2002). Therefore, it is interesting to investigate the effect of temperature on sludge reduction and microbial community structure of OSA. Notably, increasing temperature will result in additional energy consumption in a WWTP.

Fourth, studies must be performed to remove TrOCs in the effluent and residual biosolids to prevent their adverse impact on the environment and human health. One approach is to combine the wastewater or sludge treatment line of OSA with ultrasonication, ozonation, thermal treatment, bioaugmentation and other technologies that can enhance the biodegradation of TrOCs (Stevens-Garmon *et al.*, 2011). Another approach is to upgrade the main aeration tank of OSA with a membrane filtration unit to form an MBR. Depending on various factors (*e.g.*, SRT, membrane type, and others), an MBR can achieve greater TrOC removal than CAS. Using either of these approaches will require capital investment and supplementary maintenance cost.

Fifth, the minimisation of sludge during secondary treatment can be improved by adapting technologies with greater capacity to destroy volatile solids. For instance, ozonation have potential to reduce excess sludge wastage by 100% (Semblante *et al.*, 2016). Although ozonation and other advanced oxidation processes can probably outperform OSA in terms of sludge reduction, they have considerable capital and maintenance cost. These costs could be alleviated if the energy efficiency of such technologies were improved or alternative energy sources (*e.g.*, biogas from anaerobic digestion) were utilised for their operation.

9.3 REFERENCES

[1] Calace, N., Nardi, E., Petronio, B.M., Pietroletti, M. 2002. Adsorption of phenols by papermill sludges. *Environmental Pollution*, 118(3), 315-319.

[2] Semblante, G.U., Hai, F.I., Dionysiou, D.D., Fukushi, K., Price, W.E., Nghiem, L.D. 2016. Holistic sludge management through ozonation: A critical review. *Journal of Environmental Management*, 185, 79-95.

[3] Stevens-Garmon, J., Drewes, J.E., Khan, S.J., McDonald, J.A., Dickenson, E.R.V. 2011. Sorption of emerging trace organic compounds onto wastewater sludge solids. *Water Research*, 45(11), 3417-3426.

Appendix A: Supplementary figures

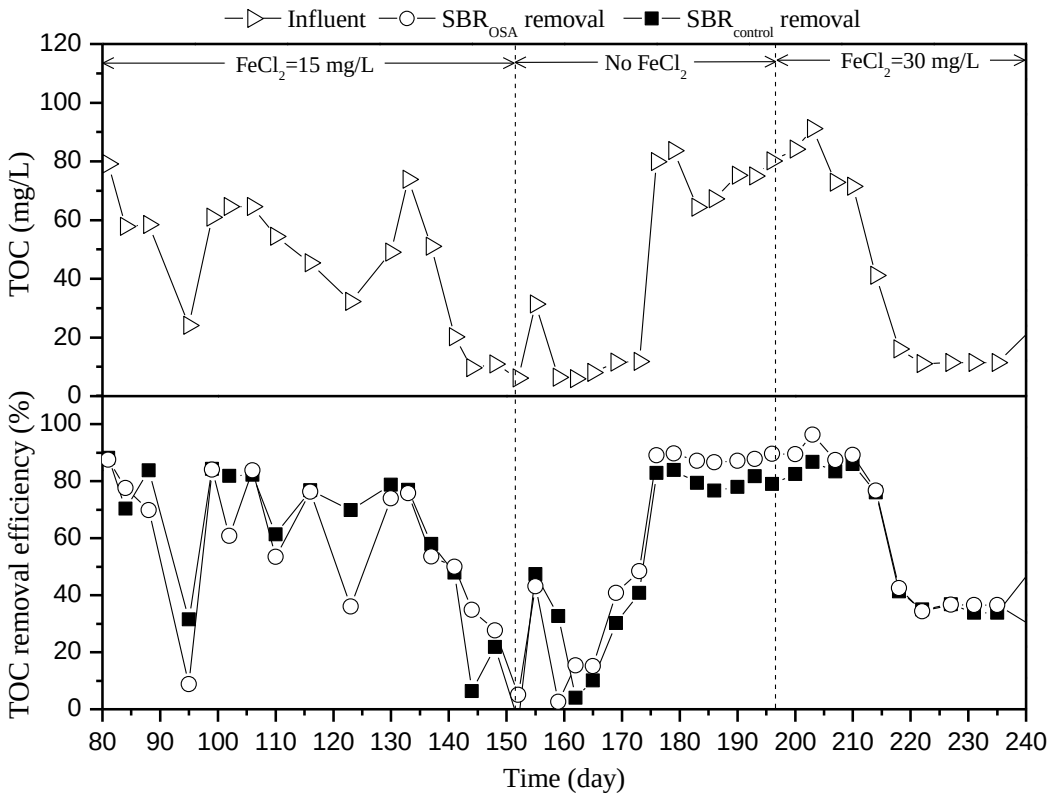


Figure A.1. TOC concentration and removal of SBR_{OSA} and $SBR_{control}$ at different dosages of $FeCl_2$ to the influent (settled domestic sewage). The SIR of OSA was 16.5% and SRT_{ext} was 20 d. The dashed lines indicate change in $FeCl_2$ dosage.

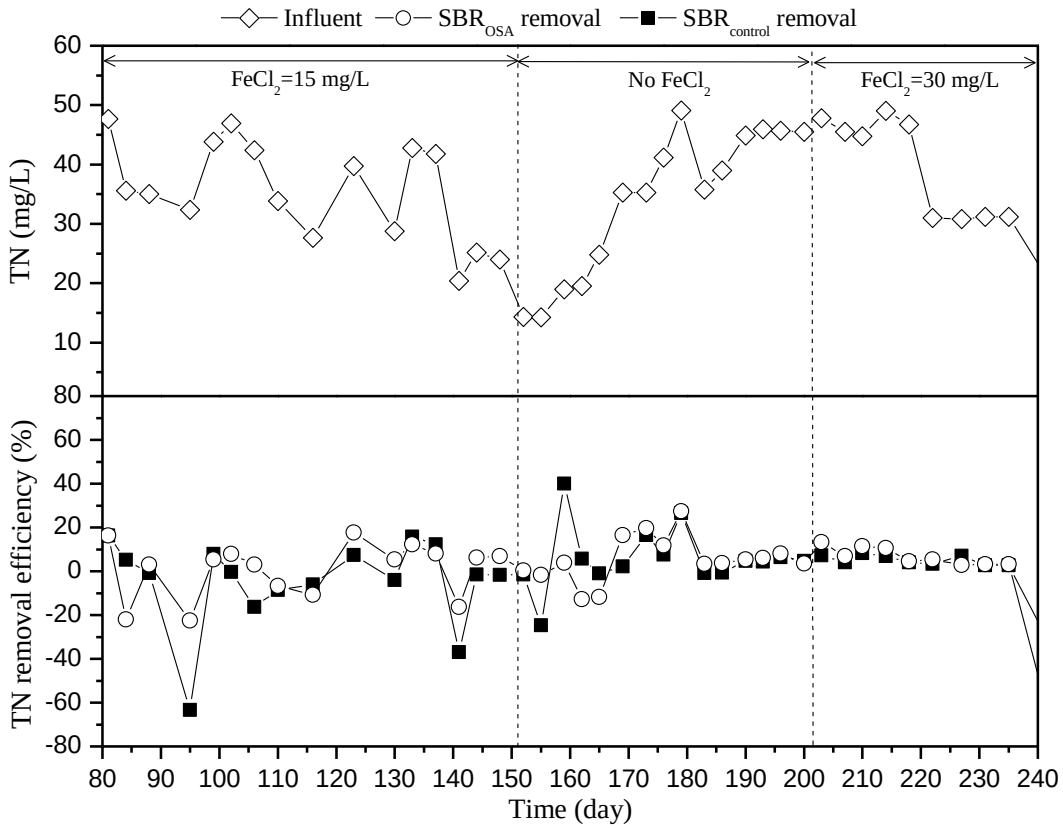


Figure A.2. TN concentration and removal of SBR_{OSA} and $SBR_{control}$ at different dosages of $FeCl_2$ to the influent (settled domestic sewage). The SIR of OSA was 16.5% and SRT_{ext} was 20 d. The dashed lines indicate change in $FeCl_2$ dosage.

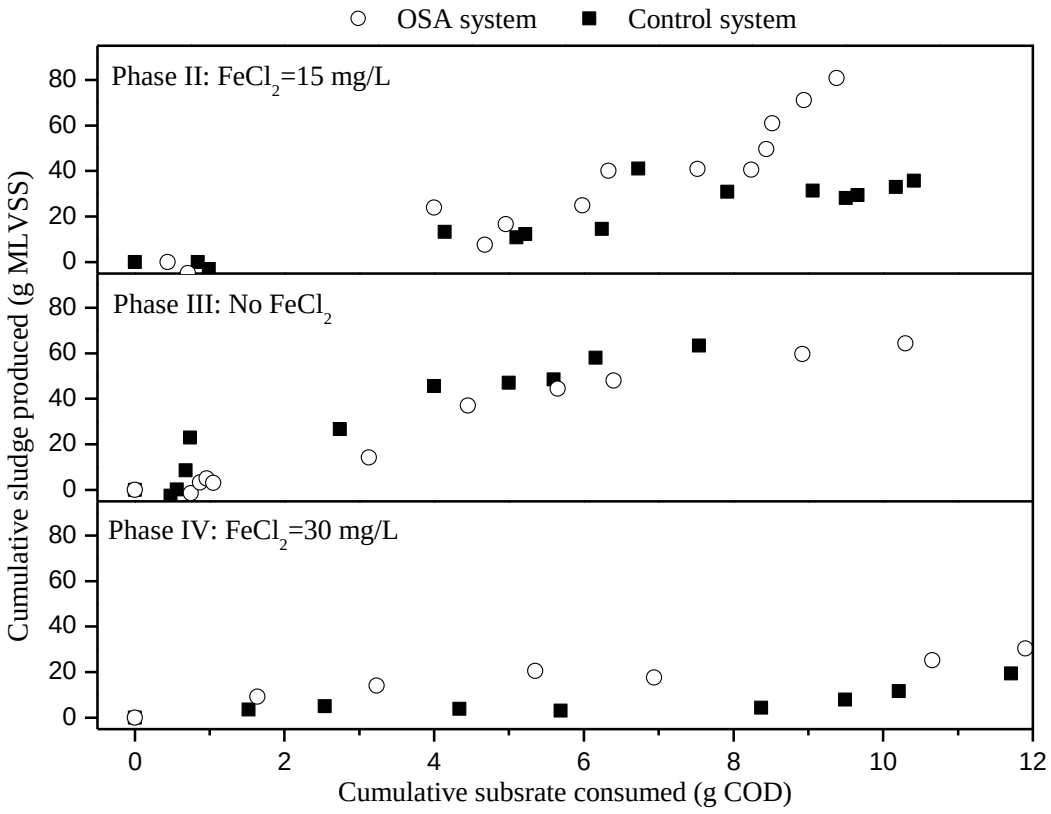


Figure A.3. Cumulative sludge produced (g MLVSS) versus cumulative substrate consumed (g COD) of the OSA (combined SBR_{OSA} and external aerobic/anoxic and anoxic reactors) and control (combined $SBR_{control}$ and aerobic digester) systems at different dosages of $FeCl_2$ to the influent (settled domestic sewage). The SIR of OSA was 16.5% and SRT_{ext} was 20 d.

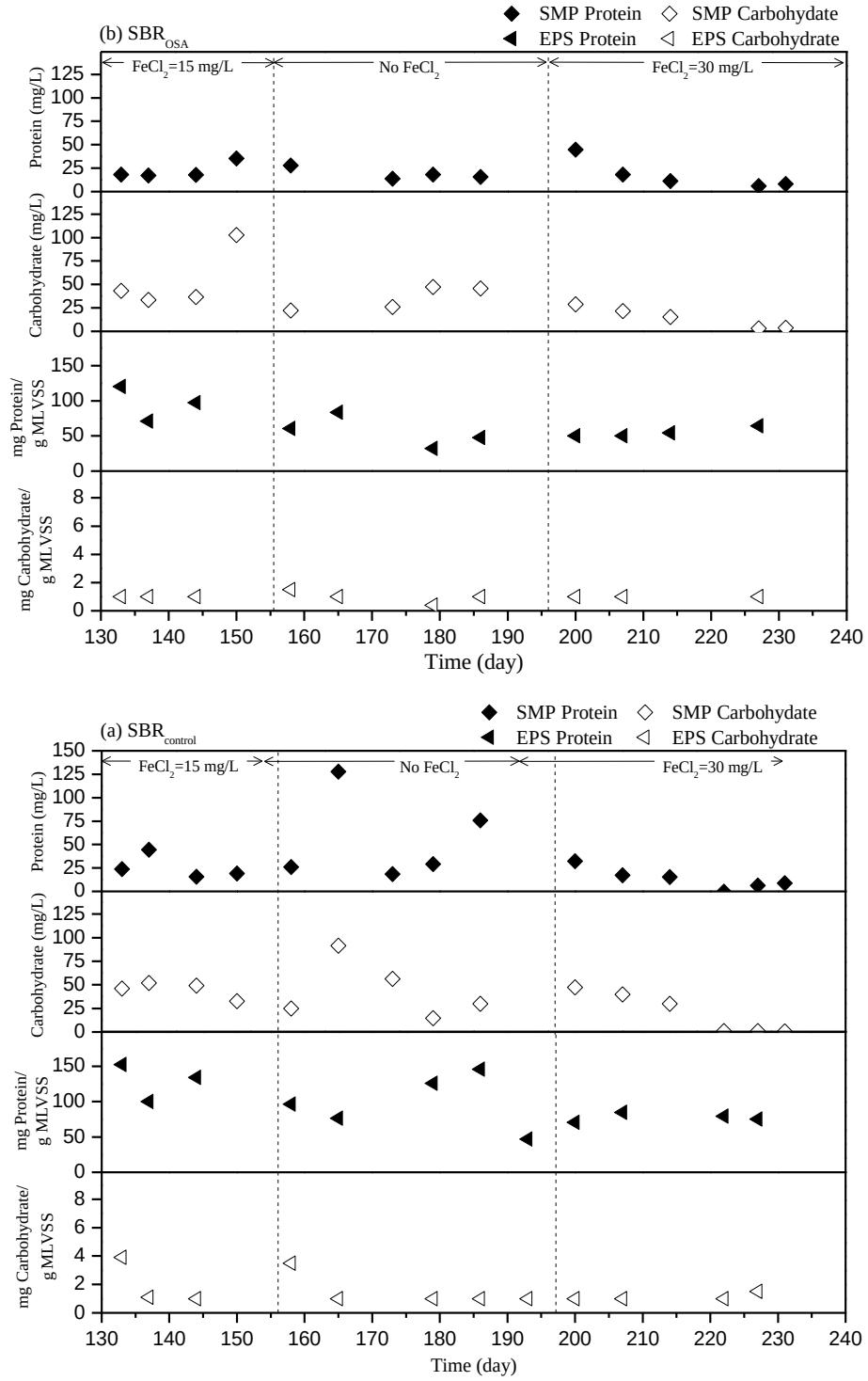


Figure A.4. Iron-associated EPS and SMP of (a) SBR_{OSA} and (b) $SBR_{control}$ at different dosages of $FeCl_2$ to the influent (settled domestic sewage). The SIR of OSA was 16.5% and SRT_{ext} was 20 d.

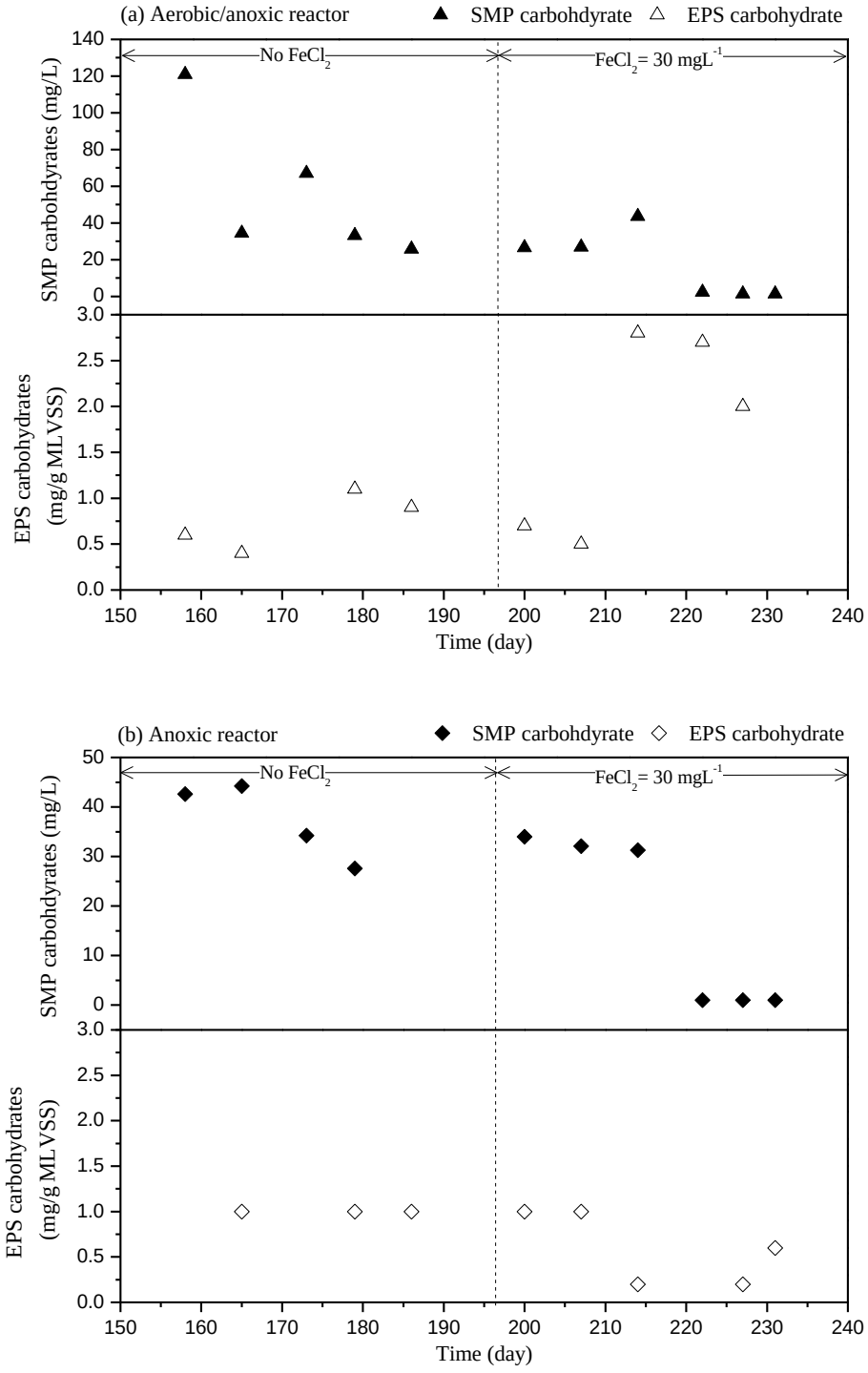


Figure A.5. Iron-associated EPS and SMP in the form of carbohydrates of the (a) aerobic/anoxic and (b) anoxic reactors of OSA when $FeCl_2$ dosage to the influent (settled domestic sewage) was zero (Phase III) and 30 mg/L (Phase IV). The SIR of OSA was 16.5% and SRT_{ext} was 20 d.

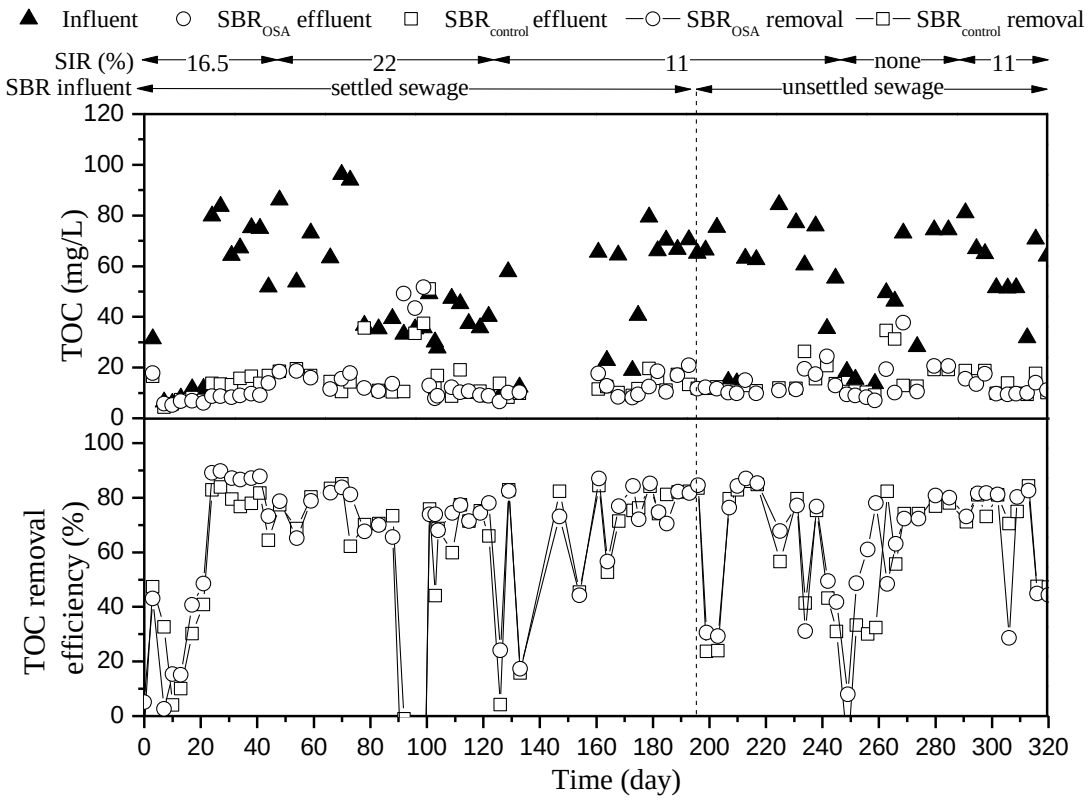
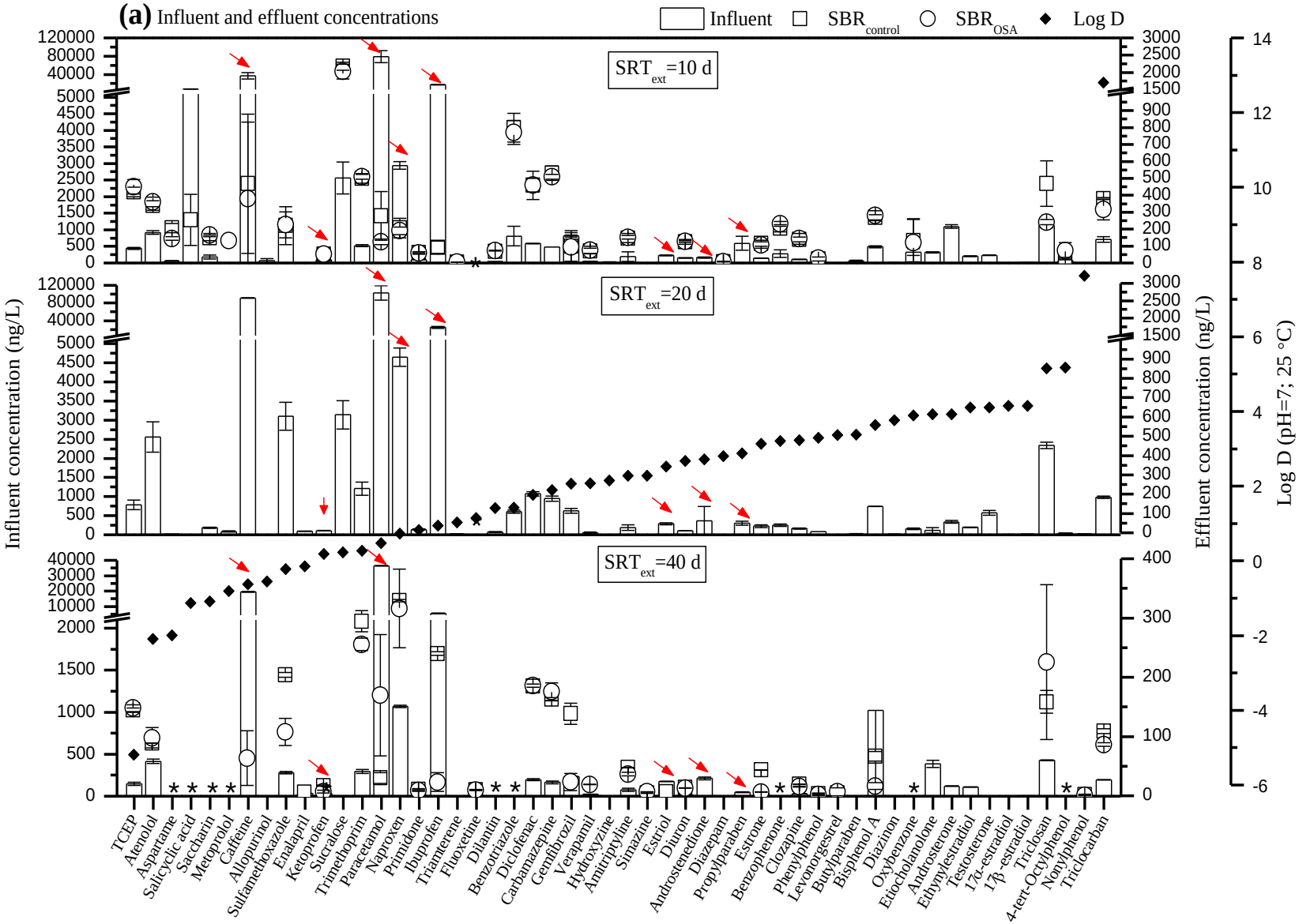


Figure A.6. TOC concentration and removal efficiency of SBR_{OSA} and $SBR_{control}$ and at different SIR (none-22%) and influent (settled and unsettled sewage). SRT_{SBR} was maintained 10 d, SRT_{ext} was maintained at 20 d, and $FeCl_2$ was not added to the influent. The dashed line indicates the change of influent from settled to unsettled sewage.


(a) Influent and effluent concentrations
Influent
SBR control
SBR OSA
Log D
SRT ext =10 d
SRT ext =20 d
SRT ext =40 d
Influent concentration (ng/L)
Effluent concentration (ng/L)
Log D (pH=7; 25 °C)
TCEP
Atenolol
Aspartame
Salicyclic acid
Saccharin
Metoprolol
Caffeine
Allopurinol
Sulfamethoxazole
Enalapril
Ketoprofen
Sucralose
Trimethoprim
Paracetamol
Naproxen
Primidone
Ibuprofen
Triamterene
Fluoxetine
Dilantin
Benzotriazole
Diclofenac
Carbamazepine
Gemfibrozil
Verapamil
Hydroxyzine
Amitriptyline
Simazine
Estriol
Diuron
Androstenedione
Diazepam
Propylparaben
Estrone
Benzophenone
Clozapine
Phenylphenol
Levonorgestrel
Butylparaben
Bisphenol A
Diazinon
Oxybenzone
Etiocholanolone
Androsterone
Ethynylestradiol
Testosterone
17α-estradiol
17β-estradiol
Triclosan
4-tert-Octylphenol
Nonylphenol
Triclocarban

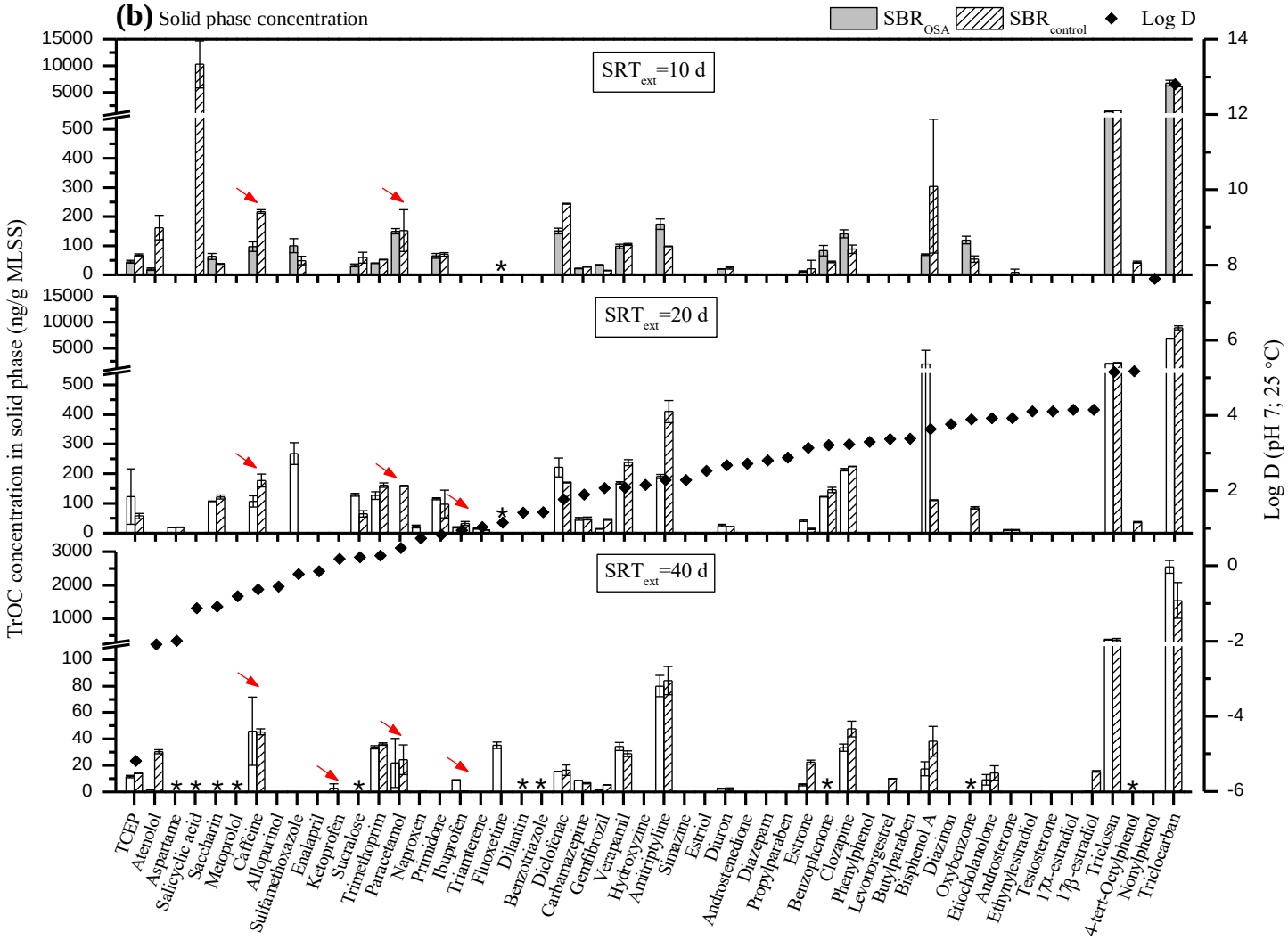
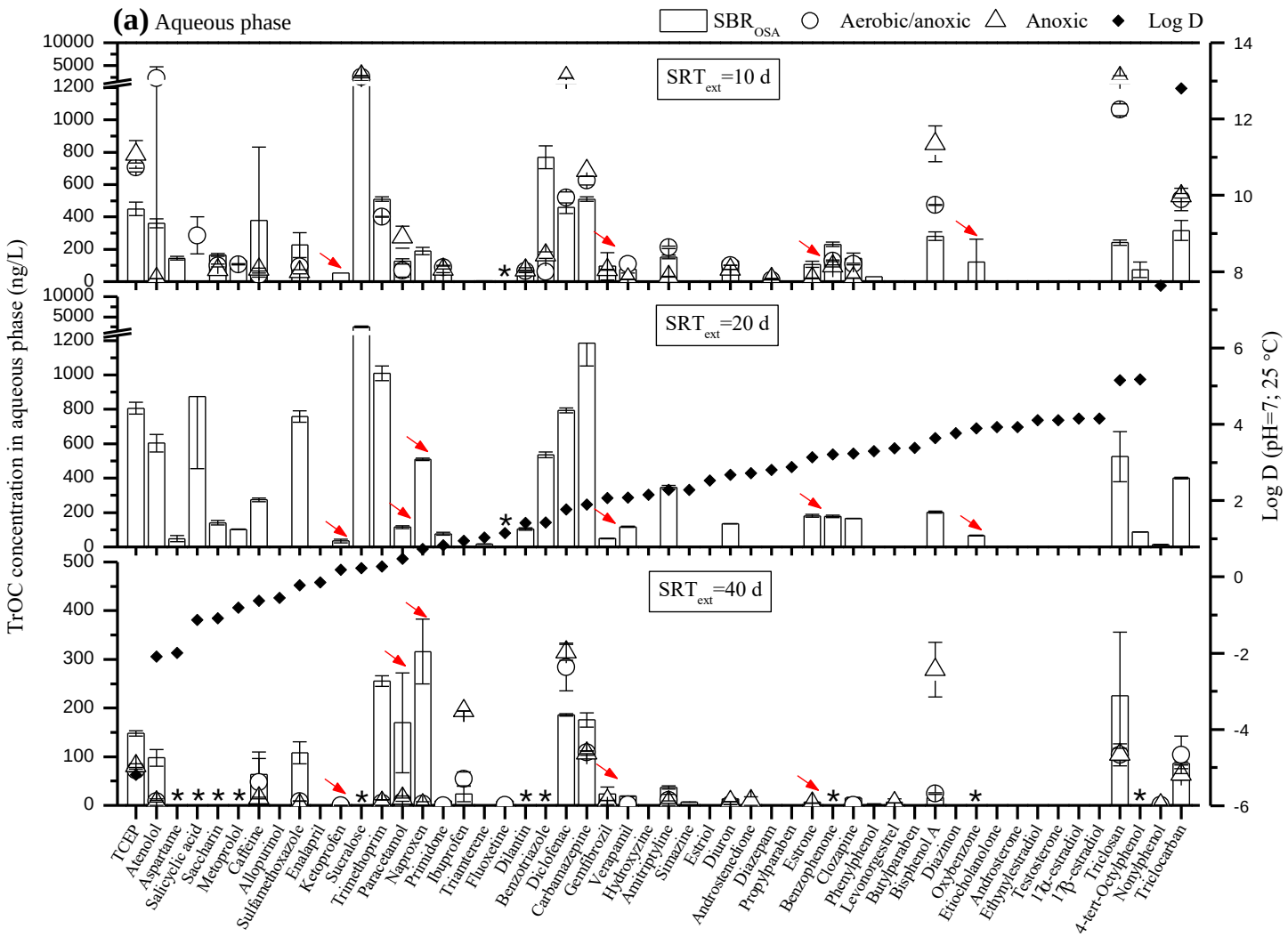


Figure A.7. TrOC concentrations in the (a) influent, effluent, and (b) solid phase of sludge of SBR_{OSA} and $SBR_{control}$ when SRT_{ext} was varied (10-40 d), SRT_{SBR} was maintained at 10 d and, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage). The values are the average of two measurements (n=2). The asterisks (*) represent TrOCs that were not analysed in a particular sampling campaign. The arrows (→) denote contaminants that were highly biodegraded.


(a) Aqueous phase
SBR$_{OSA}$
Aerobic/anoxic
Anoxic
Log D
SRT$_{ext}$=10 d
SRT$_{ext}$=20 d
SRT$_{ext}$=40 d
TrOC concentration in aqueous phase (ng/L)
Log D (pH=7; 25 °C)
TCEP
Atenolol
Aspartame
Salicylic acid
Saccharin
Metoprolol
Caffeine
Allopurinol
Sulfamethoxazole
Enalapril
Ketoprofen
Sucralose
Trimethoprim
Paracetamol
Naproxen
Primidone
Ibuprofen
Triamterene
Fluoxetine
Dilantin
Benzotriazole
Diclofenac
Carbamazepine
Gemfibrozil
Verapamil
Hydroxyzine
Amitriptyline
Simazine
Estriol
Diuron
Androstenedione
Diazepam
Propylparaben
Estrone
Benzophenone
Clozapine
Phenylphenol
Levonorgestrel
Butylparaben
Bisphenol A
Diazinon
Oxybenzone
Etiocholanolone
Androsterone
Ethynylestradiol
Testosterone
17α-estradiol
17β-estradiol
Triclosan
4-tert-Octylphenol
Nonylphenol
Triclocarban

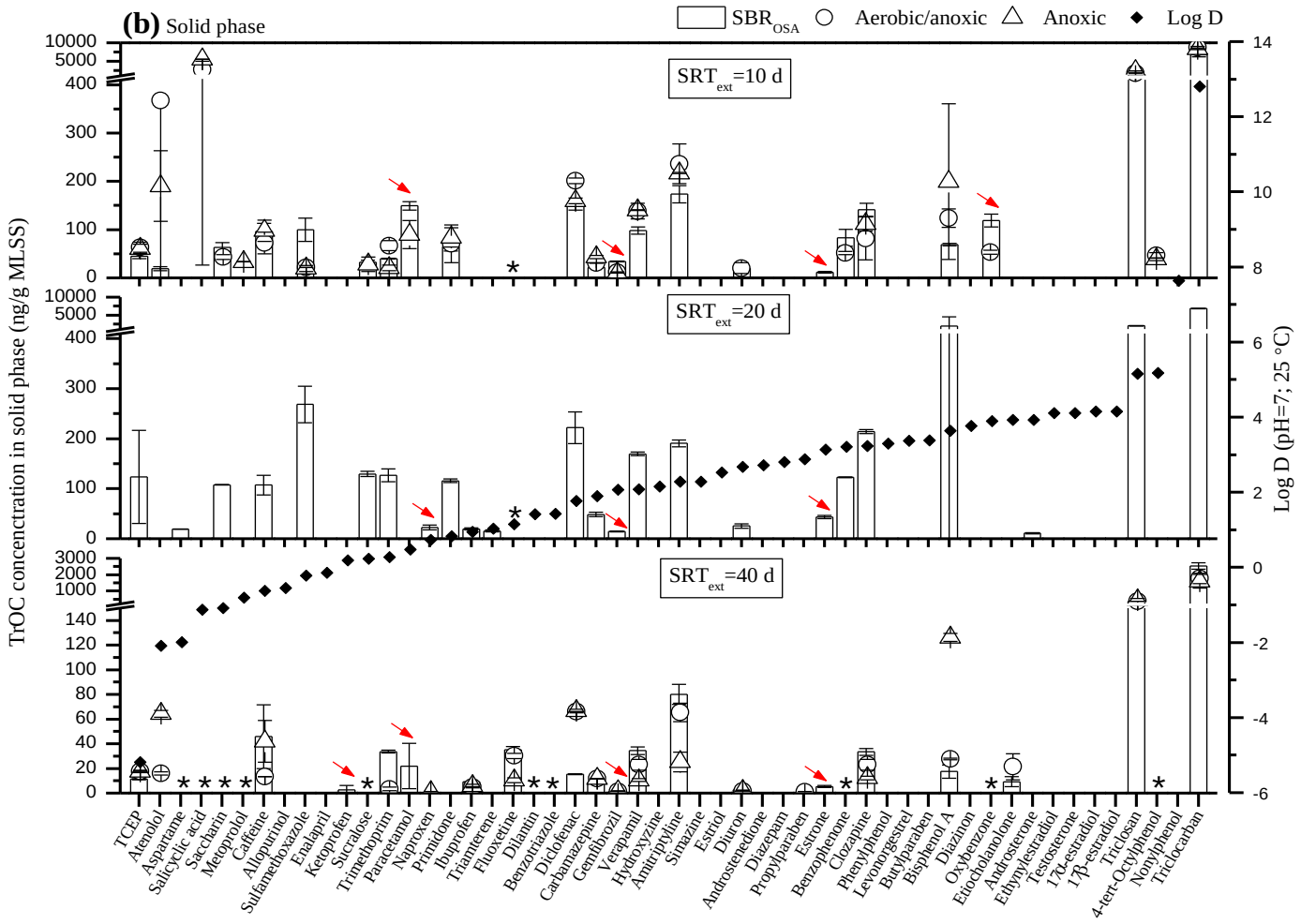
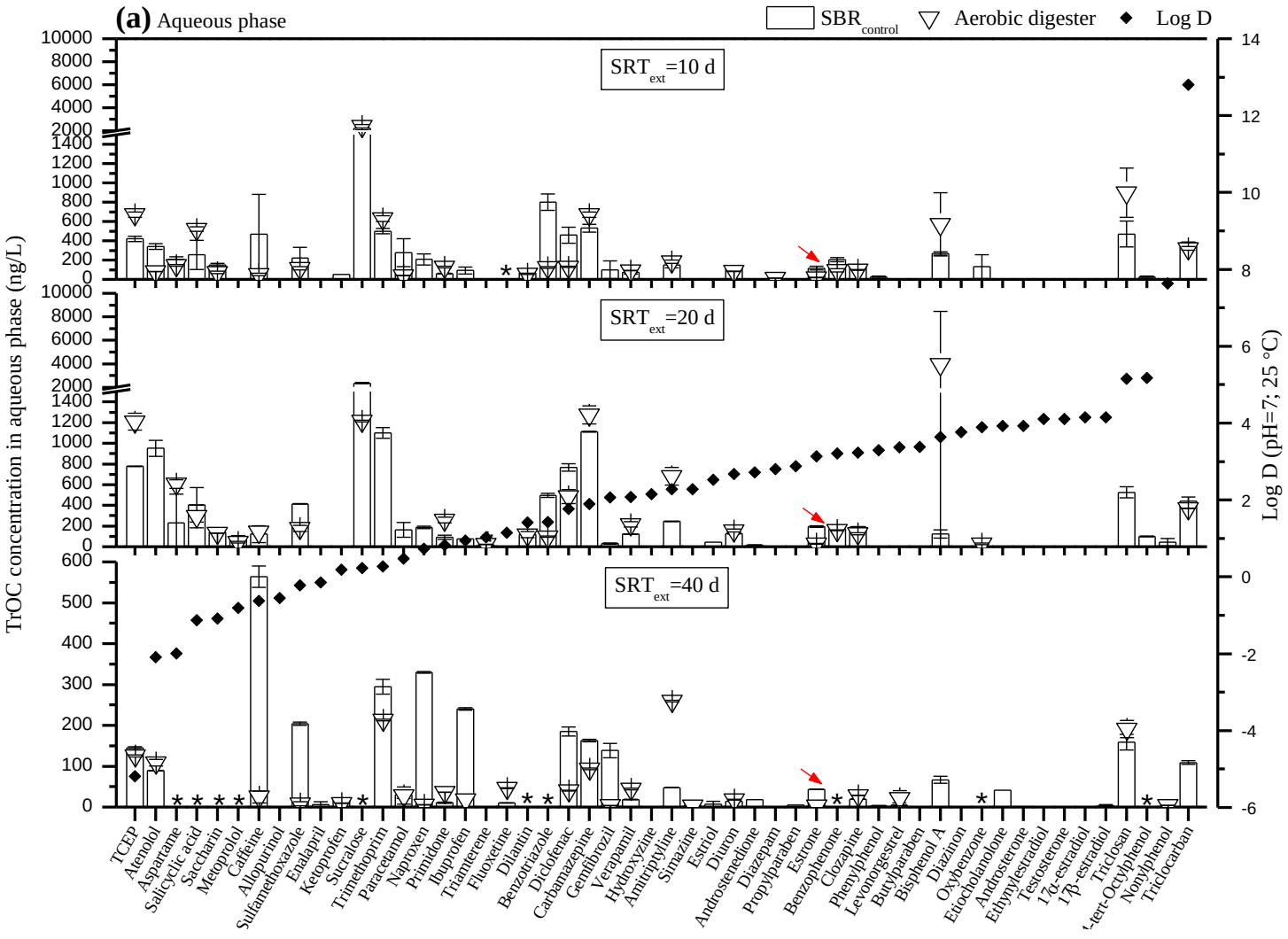


Figure A.8. TrOC concentration in the (a) aqueous and (b) solid phase of sludge in the external aerobic/anoxic and anoxic reactor of OSA when SRT_{ext} was varied (10-40 d), SRT_{SBR} was maintained at 10 d and, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage). The values are the average of two measurements (n=2). The asterisks (*) represent contaminants that were not analysed in a particular sampling campaign. The arrows (→) denote contaminants that were highly biodegraded. denote contaminants that were highly biodegraded in the aerobic/anoxic reactor. No contaminant was highly biodegraded in the anoxic reactor.


(a) Aqueous phase
SBR control
Aerobic digester
Log D
SRT ext=10 d
SRT ext=20 d
SRT ext=40 d
TrOC concentration in aqueous phase (ng/L)
Log D (pH=7; 25 °C)
TCEP
Atenolol
Aspartame
Salicylic acid
Saccharin
Metoprolol
Caffeine
Allopurinol
Sulfamethoxazole
Enalapril
Ketoprofen
Sucralose
Trimethoprim
Paracetamol
Naproxen
Primidone
Ibuprofen
Triamterene
Fluoxetine
Dilantin
Benzotriazole
Diclofenac
Carbamazepine
Gemfibrozil
Verapamil
Hydroxyzine
Amitriptyline
Simazine
Estriol
Diuron
Androstenedione
Diazepam
Propylparaben
Estrone
Benzophenone
Clozapine
Phenylphenol
Levonorgestrel
Butylparaben
Bisphenol A
Diazinon
Oxybenzone
Etiocholanolone
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Nonylphenol
Triclocarban

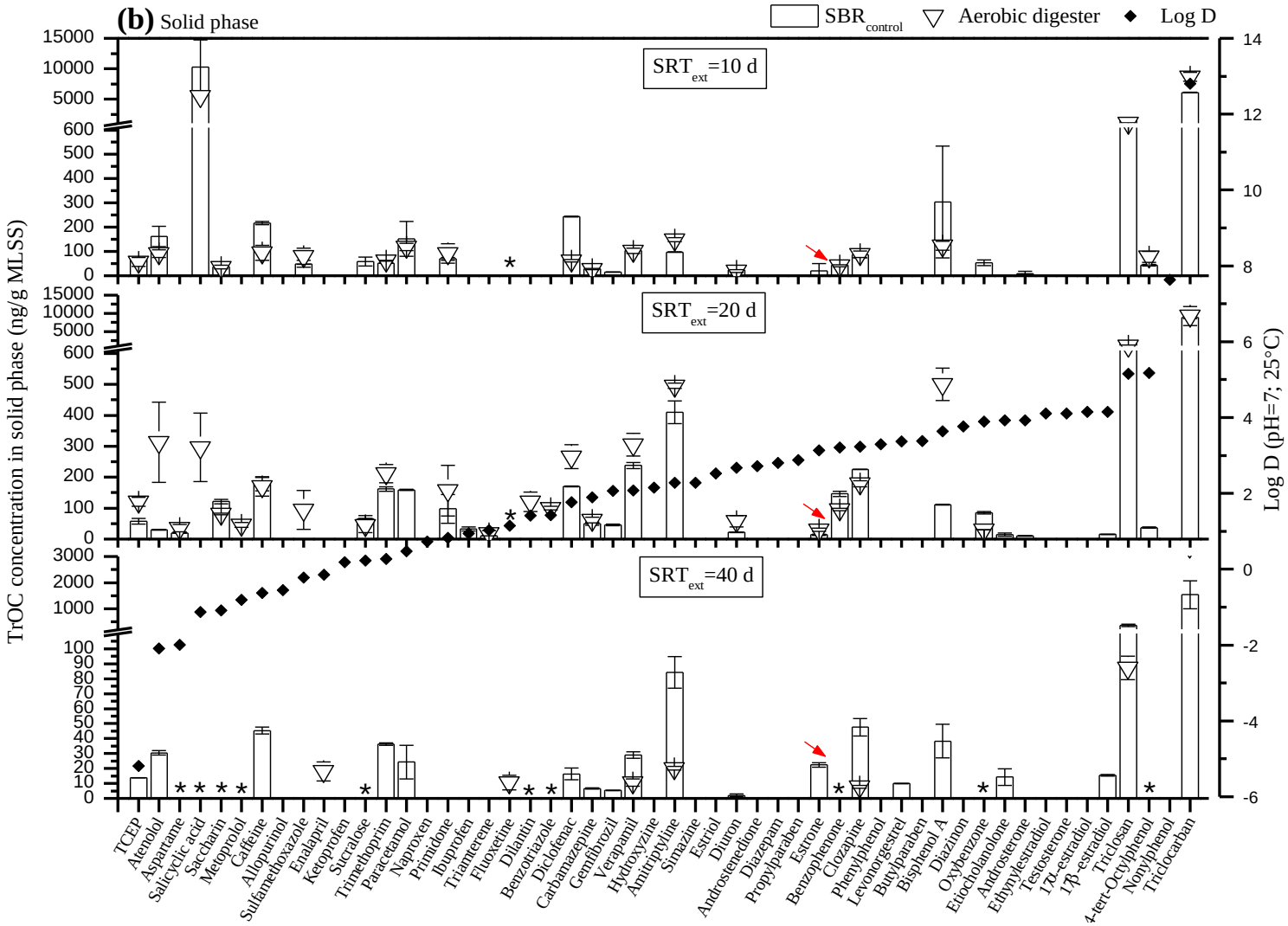


Figure A.9. TrOC concentration in the (a) aqueous and (b) solid phase of sludge in the aerobic digester when SRT_{ext} was varied (10-40 d), SRT_{SBR} was maintained at 10 d and, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage). The values are the average of two measurements (*n*=2). The asterisks (*) represent contaminants that were not analysed in a particular sampling campaign. The arrows (→) denote contaminants that were highly biodegraded.

Appendix B: Supplementary tables

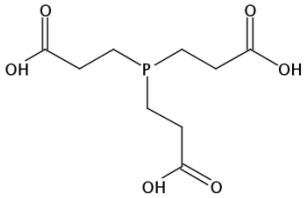
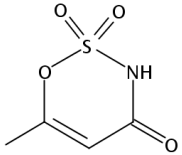
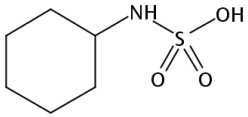
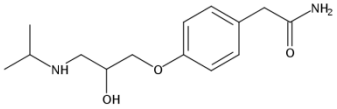
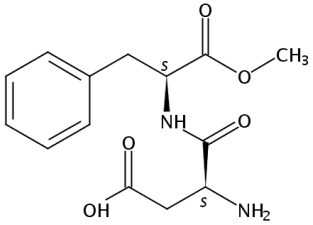
Table B.1 Sludge yield of OSA (combined SBR_{OSA} and external aerobic/anoxic and anoxic reactors) and control (combined $SBR_{control}$ and aerobic digester) systems at different SIR (none-22%) and influent (settled and unsettled sewage). SRT_{SBR} was maintained 10 d, SRT_{ext} was maintained at 20 d, and $FeCl_2$ was not added to the influent.

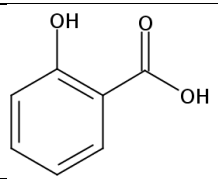
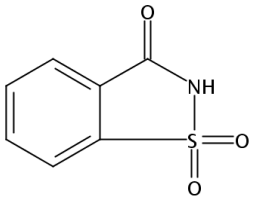
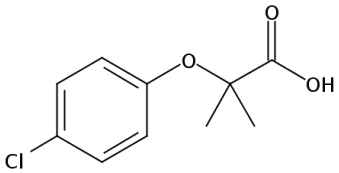
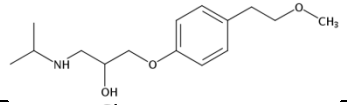
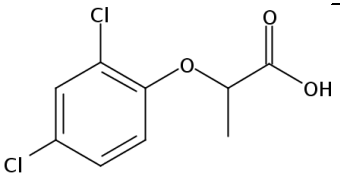
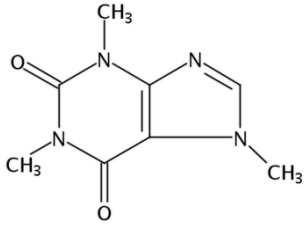
Experimental Phase	**Influent**	**SIR of OSA (%)**	**Sludge yield Y (g MLVSS/g sCOD)**			
			OSA system	R^2	*Control system*	R^2
I	Settled sewage	16.5	6.69	0.96	8.75	0.87
II	Settled sewage	22	1.95	0.57	0.96	0.65
III	Settled sewage	11	~0	-	1.23	0.88
IV	Unsettled sewage	11	0.58	0.92	1.06	0.75
V	Unsettled sewage	0	0.189	0.51	0.129	0.61
VI	Unsettled sewage	11	1.31	0.92	1.92	0.90

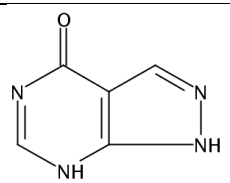
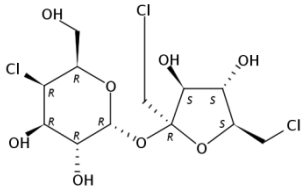
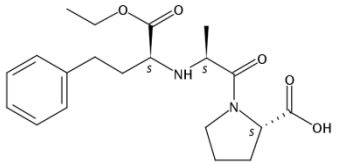
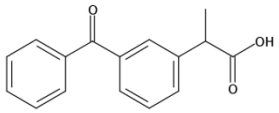
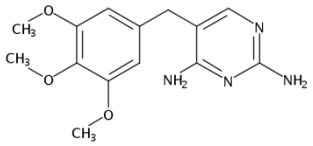
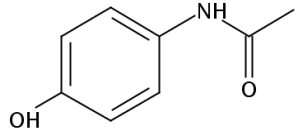
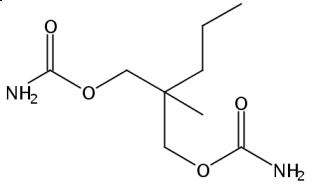
Table B.2. Sludge yield of OSA (combined SBR_{OSA} and external aerobic/anoxic,and anoxic reactors) and control (combined $SBR_{control}$ and aerobic digester) systems when $SRT_{ext.reactors}$ was varied (10-40 d)and SRT_{SBRs} was maintained at 10 d, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage).

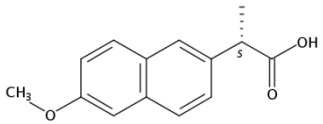
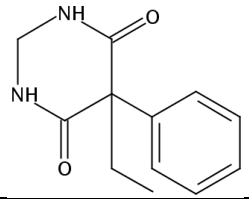
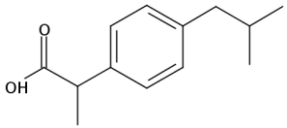
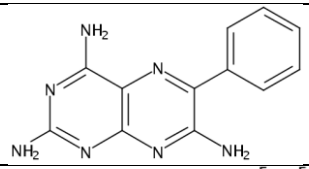
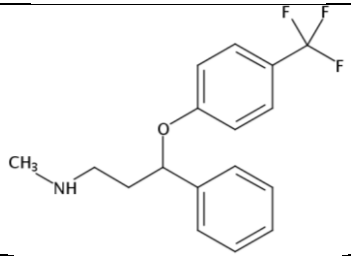
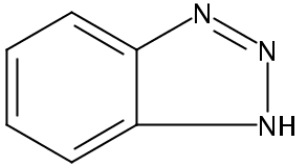
Experimental phase	**SRT_{SBR}**	**SRT_{ext}**	**Total system SRT**	**Influent tCOD concentration (mg/L)**	**Sludge yield (g MLVSS/g tCOD)**				
					Control system	*R^2*	*OSA system*	*R^2*	*Reduction (%)*
I	10	20	30	231±125 (n=13)	0.08	0.64	0.02	0.67	75
II	10	40	50	527±154 (n=19)	0.17	0.92	0.15	0.88	11
III	10	20	30	478±254 (n=12)	0.18	0.78	0.14	0.72	22
IV	10	10	20	491±194 (n=11)	0.05	0.71	0.07	0.53	None

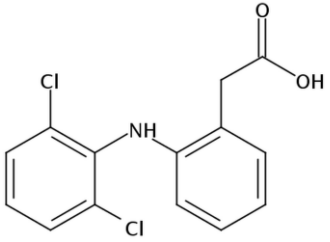
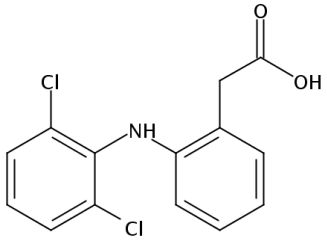
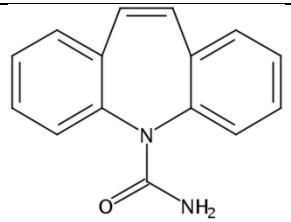
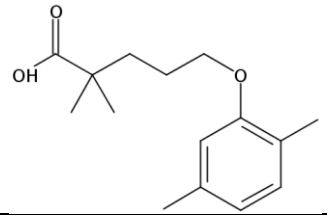
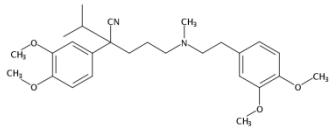
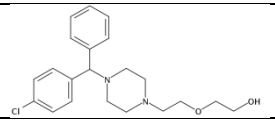
Table B.3. List of isotopically labelled standard compounds in the surrogate solution used for TrOC analysis. TrOC sampling and analysis were performed when SRT_{ext} was varied (10-40 d) and SRT_{SBRs} was maintained at 10 d, the SIR of OSA was maintained at 11%, and $FeCl_2$ was not added to the influent (unsettled sewage). The sampling campaign occurred at different seasons.

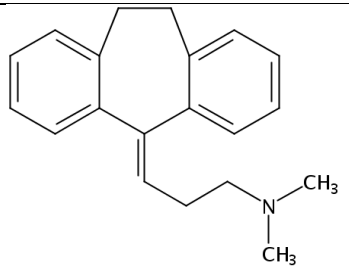
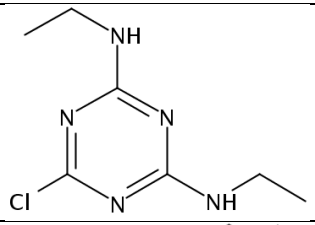
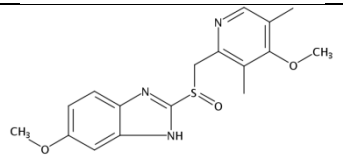
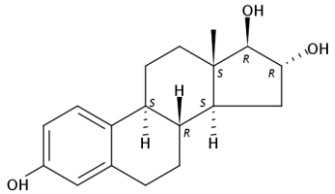
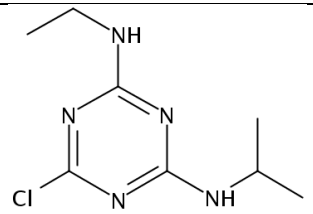
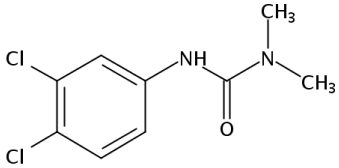
TrOC	Chemical Structure	Log D (pH 7; 25ºC)	Type or application	Detection limit (ng/L)	SRT_{ext} (d) / season					
					40 / winter		20 / spring		10 / summer	
					Analysed	Detected in influent	Analysed	Detected in influent	Analysed	Detected in influent
Tris(2-carboxyethyl) phosphene (TCEP)		-5.19	Flame retardant	5	Yes	Yes	Yes	Yes	Yes	Yes
Acesulfame		-2.88	Artificial sweetener	5	No	-	Yes	No	Yes	No
Cyclamate		-2.46	Artificial sweetener	5	No	-	Yes	No	Yes	No
Atenolol		-2.09	Pharmaceutical (beta-blocker)	5	Yes	Yes	Yes	Yes	Yes	Yes
Aspartame		-1.99	Artificial sweetener	5	No	-	Yes	Yes	Yes	Yes

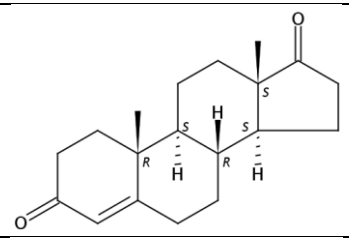
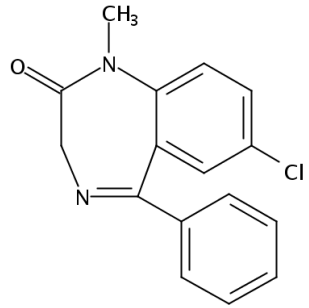
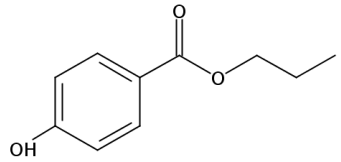
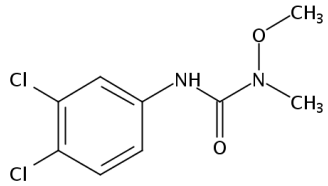
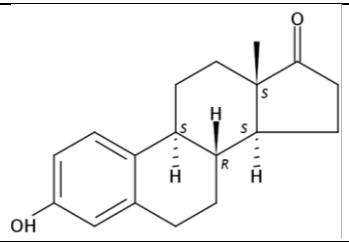
Salicylic acid		-1.13	Pharmaceutical	5	No	-	Yes	No	Yes	Yes
Saccharin		-1.09	Artificial sweetener	5	No	-	Yes	Yes	Yes	Yes
Clofibric acid		-1.06	Pesticide (herbicide)	5	No	-	Yes	No	Yes	No
Metoprolol		-0.81	Pharmaceutical (beta-blocker)	20	No	-	Yes	Yes	Yes	No
Dichloroprop		-0.77	Pesticide (herbicide)	20	No	-	Yes	Yes	No	No
Caffeine		-0.63	Food product (Stimulant)	5	Yes	Yes	Yes	Yes	Yes	Yes

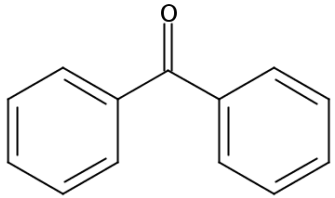
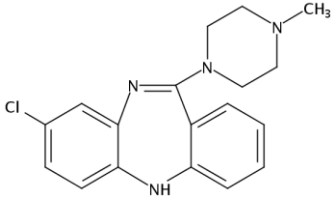
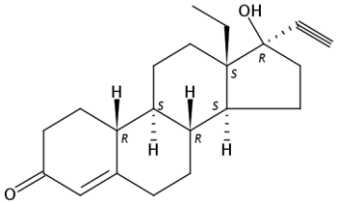
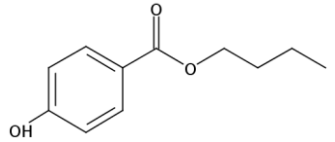
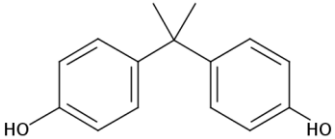
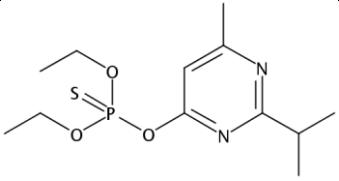
Allopurinol		-0.55	Pharmaceutical (antidiuretic)	5	No	-	Yes	No	Yes	Yes
Sucralose		-0.23	Artificial sweetener	5	No	-	Yes	Yes	Yes	Yes
Enalapril		-0.14	Pharmaceutical (angiotensin converting enzyme inhibitor)	5	Yes	Yes	Yes	No	Yes	No
Ketoprofen		0.19	Pharmaceutical (nonsteroidal anti-inflammatory drug)	20	Yes	Yes	Yes	No	Yes	Yes
Trimethoprim		0.27	Antibiotic	20	Yes	Yes	Yes	Yes	Yes	Yes
Paracetamol		0.47	Pharmaceutical	20	Yes	Yes	Yes	Yes	Yes	Yes
Meprobamate		0.70	Pharmaceutical (tranquiliser)	5	Yes	No	No	-	No	-

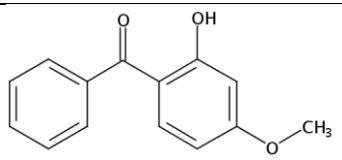
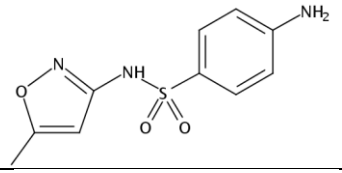
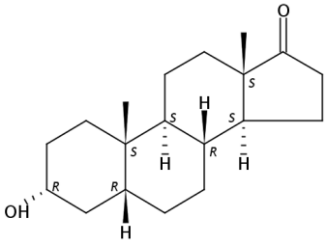
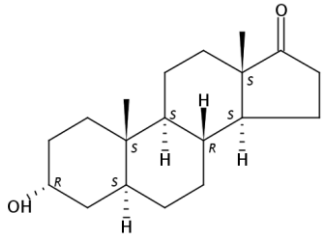
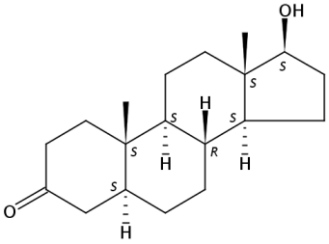
Naproxen		0.73	Pharmaceutical (nonsteroidal anti-inflammatory drug)	5	Yes	Yes	Yes	Yes	Yes	Yes
Primidone		0.83	Pharmaceutical (anticonvulsant)	20	Yes	Yes	Yes	Yes	Yes	Yes
Ibuprofen		0.94	Pharmaceutical (nonsteroidal anti-inflammatory drug)	20	Yes	Yes	Yes	Yes	Yes	Yes
Triamterene		1.03	Pharmaceutical (diuretic)	20	Yes	No	Yes	Yes	Yes	Yes
Fluoxetine		1.15	Pharmaceutical (antidepressant)	5	Yes	Yes	No	-	No	-
Benzotriazole		1.42	Industrial anticorrosive	20	No	-	Yes	Yes	Yes	Yes

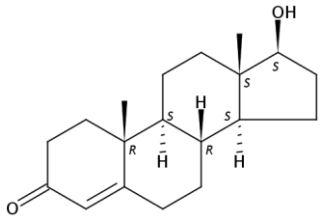
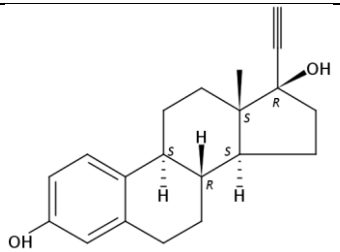
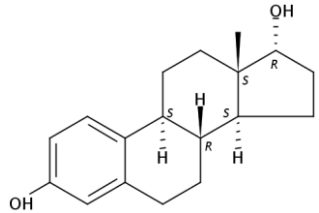
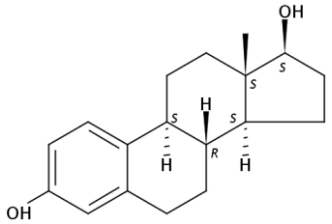
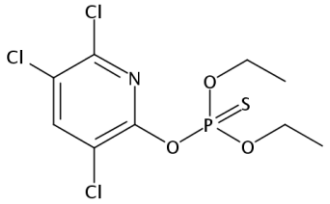
Diclofenac		1.77	Pharmaceutical (nonsteroidal anti-inflammatory drug)	20	Yes	Yes	Yes	Yes	Yes	Yes
Phenylphenol		1.88	Pesticide (fungicide)	5	Yes	Yes	Yes	No	Yes	Yes
Carbamazepine		1.89	Pharmaceutical (anticonvulsant and analgesic)	5	Yes	Yes	Yes	Yes	Yes	Yes
Gemfibrozil		2.07	Pharmaceutical (cholesterol and triglyceride reducer)	5	Yes	Yes	Yes	Yes	Yes	Yes
Verapamil		2.08	Pharmaceutical (calcium channel blocker)	5	Yes	Yes	Yes	Yes	Yes	Yes
Hydroxyzine		2.15	Pharmaceutical (antihistamine)	5	Yes	No	Yes	No	Yes	Yes

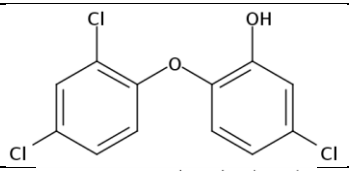
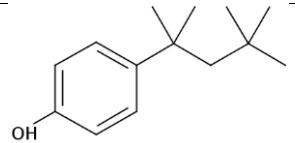
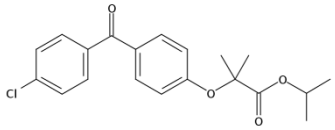
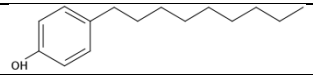
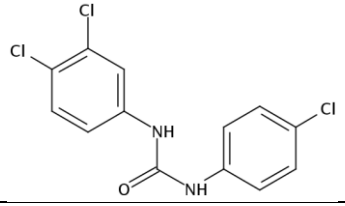
Amitriptyline		2.28	Pharmaceutical (antidepressant)	20	Yes	Yes	Yes	Yes	Yes	Yes
Simazine		2.28	Pesticide (herbicide)	5	Yes	Yes	Yes	No	Yes	No
Omeprazole		2.35	Pharmaceutical (anti-gastroesophaeg al reflux)	20	Yes	No	Yes	No	Yes	No
Estriol		2.53	Hormone	5	Yes	Yes	Yes	Yes	Yes	Yes
Atrazine		2.64	Pesticide (herbicide)	5	Yes	No	Yes	No	Yes	No
Diuron		2.68	Pesticide (herbicide)	5	Yes	Yes	Yes	Yes	Yes	Yes

Androstenedione		2.72	Hormone	5	Yes	Yes	Yes	Yes	Yes	Yes
Diazepam		2.80	Pharmaceutical (muscle relaxant)	5	Yes	No	Yes	No	Yes	Yes
Propylparaben		2.88	Personal care product formulation	20	Yes	Yes	Yes	Yes	Yes	Yes
Linuron		3.12	Pesticide (herbicide)	5	Yes	No	Yes	No	Yes	No
Estrone		3.13	Hormone	5	Yes	Yes	Yes	Yes	Yes	Yes

Benzophenone		3.21	UV filter	5	No	-	Yes	Yes	Yes	Yes
Clozapine		3.23	Pharmaceutical (antipsychosis)	5	Yes	Yes	Yes	Yes	Yes	Yes
Levonorgestrel		3.37	Hormone	5	Yes	Yes	Yes	No	Yes	No
Butylparaben		3.38	Personal care product formulation	20	No	-	Yes	Yes	Yes	Yes
Bisphenol A		3.64	Industrial chemical (plastic production)	5	Yes	Yes	Yes	Yes	Yes	Yes
Diazinon		3.77	Pesticide	5	Yes	No	Yes	Yes	Yes	Yes

Oxybenzone		3.89	UV filter	20	No	-	Yes	Yes	Yes	Yes
Sulfamethoxazole		3.90	Antibiotic	20	Yes	Yes	Yes	Yes	Yes	Yes
Etiocholanolone		3.93	Hormone	5	Yes	Yes	No	-	No	-
Androsterone		3.93	Hormone	5	Yes	Yes	Yes	Yes	Yes	Yes
Di-hydrotestosterone		3.93	Hormone	5	Yes	No	Yes	Yes	Yes	Yes

Testosterone		4.11	Hormone	5	Yes	No	Yes	Yes	Yes	Yes
Ethynylestradiol		4.11	Xenoestrogen	5	Yes	Yes	Yes	Yes	Yes	Yes
17α-estradiol		4.15	Hormone	5	Yes	No	Yes	Yes	Yes	No
17β-estradiol		4.15	Hormone	5	Yes	Yes	Yes	Yes	Yes	Yes
Chlorpyrifos		5.00	Pesticide	5	No	-	Yes	No	Yes	No

Triclosan		5.15	Antibiotic	20	Yes	Yes	Yes	Yes	Yes	Yes
4-tert-Octylphenol		5.18	Industrial surfactant	5	No	-	Yes	Yes	Yes	Yes
Fenofibrate		5.80	Pharmaceutical (cholesterol and triglyceride reducer)	20	No	-	Yes	Yes	Yes	Yes
Nonylphenol		7.63	Detergent	10	Yes	Yes	Yes	Yes	Yes	No
Triclocarban		12.80	Industrial surfactant	20	Yes	Yes	Yes	Yes	Yes	Yes